TARGETED DELIVERY IN VITRO FROM MAGNETIC VESICLE GELS

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Abstract

Membrane sacs, known as vesicles and liposomes have been widely used as stores for bioactive materials both \textit{in vitro} and \textit{in vivo}. The vesicles are biocompatible and \textit{in vitro} experiments often use them in conjunction with magnetic nanoparticles. The magnetic nanoparticles allow the liposomes to be magnetically located and act as a trigger for release of the encapsulated materials. However, these magnetic vesicles or ‘magnetoliposomes’ as they are also known have not managed to cross the barrier into clinical use. The work in this thesis aims to develop a novel system of magnetoliposomes for use in a biological environment.

Magnetoliposomes are created from phospholipid suspensions extruded to give a spherical bilayer membrane. This membrane is doped with biotinylated lipids. These lipids are key to allowing the system to work \textit{in vitro}. The magnetic nanoparticles are formed from iron and are coated with a novel synthetic linker to allow them to interact with the liposomes. When the liposomes and the nanoparticles are mixed in the presence of the protein avidin, large heirarchacal structures are formed which are stable under physiological conditions.

The magnetoliposomes are held in an alginate hydrogel scaffold which acts as a support for the liposomes and as an adherent cell scaffold for tissue culture. This work demonstrates that this system can be used to encapsulate and release a range of bioactive molecules such as nickel chloride as a mimic for cytotoxic cancer drugs, ascorbic acid-2-phosphate for the upregulation of collagen production in chondrocytes and SB 431542 for the differentiation of mouse embryonic stem cells.

The results shown in this work demonstrate that it is possible to use this novel linking system to create a new form of magnetoliposomes which are stable, biocompatible and easy to form and use. This work also demonstrates a strong model for possible drug delivery \textit{in vivo}. 

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List of Abbreviations

acac      acetylacetone
ADP       adenosine diphosphate
AMAC      2-aminoacridone
AMF       alternating magnetic field
ATP       adenosine triphosphate
BBN       bombasin
BMSC      bone marrow stromal cell
BSA       bovine serum albumin
DAPI      4,6-diamidino-2-phenylindole
DC8.9PC   1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine
DCP       dihexadecyl phosphate
DHPE      1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine
DIPEA     N,N-Diisopropylethylamine
DLS       dynamic light scattering
DMEM      Dulbecco’s modified Eagle’s medium
DMF       N,N-Dimethylformamide
DMPC      1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPG      1,2-dimyristoyl-sn-glycero-3-phosphoglycerol
DMSO      dimethyl sulfoxide
DNA       deoxyribonucleic acid
DODAC     N,N-dioleyl-N,N-dimethylammonium chloride
DOGS-NTA  1,2-dioleoyl-sn-glycero-3-[N-(5-amino-1-carboxypentyl)iminodiacetic acid]
DOPC      1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE      1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP     1,2-dioleoyl-3-trimethylammonium-propane
DPPC      1,2-dipalmitoyl-3-sn-glycerophosphatidylecholine
DPPE      1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
dsPC      1,2-distearoyl-sn-glycero-3-phosphocholine
dsPE      1,2-distearoyl-sn-glycero-3-phosphoethanolamine
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPC</td>
<td>egg phosphocholine</td>
</tr>
<tr>
<td>ESEM</td>
<td>variable pressure electron scanning microscopy</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GADPH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl</td>
</tr>
<tr>
<td>NIPAM</td>
<td>poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>OBG</td>
<td>n-octyl-D-glucoside</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>PEO</td>
<td>polyethyleneoxide</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>POPG</td>
<td>1-palmitoyl-2-oleyl-sn-glycero-3-phospho-(1-rac-glycerol)</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinylalcohol</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid peptide sequence</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SB 431542</td>
<td>4-(5-benzol(1,3)dioxol-5-yl-4-pyridin-2-yl-1H-imidazol-2-yl)-benzamide hydrate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TCP</td>
<td>tissue culture plastic</td>
</tr>
<tr>
<td>TNE</td>
<td>Tris (50 mM), NaCl (100 mM), EDTA (0.1 mM)</td>
</tr>
</tbody>
</table>
$T_1$ Longitudinal relaxation time

$T_2$ Transverse relaxation time

*This thesis is dedicated to Dom who is my eagle and to Ruth who really was worth it all*
Acknowledgments

Firstly I would like to thank my supervisors. Throughout the last 4 years Dr Julie Gough has remained completely unflappable and has administered support, kindness, and very sound advice on everything from cell culture to childbirth. Without her this thesis would never have been written. Dr Simon Webb has given invaluable advice. His guidance and ideas when entering new fields have allowed the multidisciplinary nature of this project to develop. His interest and drive in this project have led to some of the most interesting results. Thank you.

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Finally thanks to the staff at the NNUH who patched me up and kept getting me back on my feet throughout 2011, to my wonderful family, and to Dom without whose support none of my achievements in the last 8 years would have been obtained.
Rationale for submitting a thesis in alternative format

For two main reasons I have decided to submit my thesis in alternative format. Firstly, in 2009 two of my papers were published in MRS, Journal of Materials Science and Materials in Medicine under Felicity Leng and a third published in Angewandte Chemie International Edition in 2011 under Felicity de Cogan. Secondly, the rest of my thesis fits well into two main chapters, one investigating the delivery of cytotoxic drugs to cells through the medium of vesicle gels and the second exploring the possibilities of vesicle-nanoparticle assemblies in the induced differentiation of stem cells.

This thesis is structured to include a general introduction to my work and a detailed materials and methods section. This is followed by three published papers. The next chapters are unpublished results formatted as journal articles to integrate with the thesis structure. The final chapter draws conclusions from all the work presented and highlights future approaches.

I hope that submitting my thesis in this format will prepare the ground for the publication of further papers, which will aid me in the attainment of a postdoctoral position.
Chapter 1

Introduction

This chapter covers a basic introduction and literature review for all the work included in this thesis in both published and unpublished material.
Chapter 1 General Introduction

1.1 Phospholipids

Phospholipids are a group of lipids formed from two main constituents, a phosphate head group and an organic tail such as an acyl group. These components give the phospholipid an amphiphilic nature; the negatively charged phosphate head group is hydrophilic and the organic chains are hydrophobic. Phospholipids occur naturally in biological systems, commonly cell membranes, where they form bilayers surrounding the cell. They are ideal as cell membranes, as phospholipid bilayers are fluid, display elastic properties and are semi-permeable, which allows them to control membrane transport. Phospholipids have also been extensively studied in synthetic systems. They can form structures, such as vesicles (liposomes) that can mimic cells and store active molecules. Synthetic derivatives of natural lipids have also been made, such as 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC).

The ability of phospholipids to form different phases has been extensively studied. The structures most commonly formed are micelle, hexagonal, lamellar, inverse hexagonal and inverse micelle (figure 1.1). These structures are dependent on the spatial requirements of the headgroup, the hydrophilic/hydrophobic interface and arrangements of the lipid chains can determine the structure of the phase. For a monolayer or bilayer to remain planar, all of the interactions need to be in balance. Interfacial tensions occur at the hydrophilic-hydrophobic interface. Phosphate headgroups will often undergo repulsive interactions due to steric, electrostatic and hydration affects, these can be overcome to some extent by attractive interactions, such as hydrogen bonding. In an aqueous system the reverse is true and positive curvature is observed. The extent of curvature is normally dictated by the lipids present in the bilayer and is dependent on the thickness and the deformability of the bilayer. Bilayer structures are fluid, changeable structures which can exist in different phases. The phase the structure adopts is dependent on the hydration of the system, the temperature, the pressure and the lipid composition. The phases in which the membrane is observed change due to lipid orientation and the structure has different properties at each phase change (Figure 1.2). The most commonly observed phases in phospholipid bilayers are solid, gel, liquid crystalline and liquid. The gel
phases differ through the packing of the acyl chains and the hydration of the carbonyl groups.

Figure 1.1 a) phospholipid structures a) micelle and b) liposome (vesicle)

Lipid based structures have been intensively studied as drug delivery platforms where a range of structures can be formed from natural or synthetically derived compounds. Monolayer structures such as micelles were used to encapsulate hydrophobic drug molecules. Indomethacin, adriamicin and antibiotics have all been entrapped inside micelle delivery vehicles. Polyethylene glycol groups are normally used to cap the micelles in order to increase circulation time in vivo. Micelles can also be targeted and made stimuli sensitive to increase their drug delivery capabilities. Phospholipid monolayers are used as surfaces for other colloidal nanomedical devices, such as magnetic nanoparticles, in order to increase biocompatibility and biodispersity. However, the most commonly used lipid delivery vehicles are bilayer vesicles, which are dispersible in aqueous systems and have an aqueous core.

Figure 1.2 Phase diagram of phospholipid structures formed from DPPC, from Chemistry and Physics of lipids. $L_m$ is Liquid crystalline, $L_β$ is the interdigitated gel phase and $P_β$ is the first of the gel phases.
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1.2 Phospholipid Vesicles

Phospholipid vesicles or ‘liposomes’ are formed from non-toxic lipids. The size of the liposomes is dependent on the conditions used for liposomal formation, but normally ranges from 50 nm - > 20 µm. The formation conditions and lipids used also give rise to differing structures. Small unilamellar vesicles (SUVs) usually have a diameter of less than 100 nm. These consist of a single bilayer encapsulating an aqueous core, large unilamellar vesicles (LUVs) have a diameter of > 1 µm. At large sizes > 500 nm multilamellar vesicles can form, these structures have a spherical bilayer, enclosing smaller bilayer units in a concentric manner.

Further structural modifications of the liposomes have evolved in order to maximise the ability of liposomes as drug delivery vehicles. Classic liposome structures were initially used as nanocarriers as they could be used to encapsulate hydrophilic, hydrophobic and amphiphilic molecules in different areas of the liposome depending on the hydrophobic nature (Figure 1.3). One of the most important clinical aspects of liposomes in their use as drug delivery vehicles is their biodistribution, although early experiments in vivo showed that liposomes were cleared very fast from the body, longer circulation was achieved when polyethylene glycol (PEG) groups was grafted onto the external surface of the liposomes. The PEG groups shielded the liposomes from uptake by the reticularendothelial system, this allowed longer circulation times in the blood stream. Long circulating liposomes have been used in various cancer chemotherapy treatments, such as Doxil\textsuperscript{TM}. Doxil\textsuperscript{TM} is encapsulated Doxorubicin in PEG grafted liposomes and has been shown to be much more efficient than free intravenously injected Doxorubicin.
Although untargeted liposomes can often become located in tumours due to increased uptake by cancerous cells, this targeting effect is not reliably strong and does not occur in all tumours. Consequentially, to obtain high levels of targeted release, the vesicles need to be designed to target specific locations by decorating the phospholipid bilayer. Targeting groups such as proteins or antibodies can be added to vesicles either by covalent linkage to the phosphate head group or by attaching the targeting molecule to a lipid anchor. External targeting can also be used by encapsulating stimuli sensitive particles in either the aqueous core of the vesicle or the hydrophobic bilayer. These nanoscale particles can interact strongly with external stimuli such as light, sound or magnetic fields to stimulate release (Figure 1.4). This creates a complex hierarchical liposomal system, which can be used to encapsulate several different materials simultaneously and can be targeted to a specific site, e.g. a tumour using multiple methods.

1.2.1 Methods of creating phospholipid vesicles
There are several methods for creating phospholipid vesicles. The method chosen for a particular process usually depends on the requirements of the system. Some methods give very high encapsulation efficiencies of drugs, some protect the
Chapter 1 General Introduction

activation of the biomolecules they carry, others are quick and simple to follow.\textsuperscript{26,27,28} Each method has its advantages and disadvantages.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{liposome_diagram}
\caption{Formation of stimuli sensitive liposome}
\end{figure}

Liposomes have been formed using the interdigitation method, a process that involves several steps.\textsuperscript{29,30,31} Lipids are dissolved in high concentrations of ethanol followed by the addition of buffer and the mixture vigorously vortexed. Liposomes formed using this method often exhibit high encapsulation efficiency and have good size distribution. Polozova \textit{et al} showed that liposomes were created using this method with high levels of homogeneity (90 \%) displayed a diameter of 350 nm.\textsuperscript{31} Liposomes could also be prepared using the hydration method. This method involves lipids being dissolved in chloroform and the solution removed under pressure, creating a thin lipid film.\textsuperscript{32,33,34} The film is then hydrated using aqueous medium and heated above lipid transition temperature for 30 minutes, then cooled for 20 minutes. The lipid film is equilibrated to optimum pH, then incubated for a further 20 minutes. The lipid mixture is then equilibrated again at room temperature for a further 20 minutes, then homogenised. This method gives high encapsulation efficiency, a narrow size distribution and predominantly unilamellar vesicles, but is very time consuming.
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A freeze/thaw process is commonly used for encapsulating drugs. Lipids are dissolved in 1:1 chloroform:methanol, and then dehydrated under reduced pressure and dried at 0 mmHg overnight. The lipid film is reconstituted in aqueous medium and subjected to 10 freeze/thaw cycles, as the samples are frozen in liquid nitrogen and then thawed at 60 °C. The liposomes are then extruded at 80 °C, 15 times through a specific pore sized membrane. This method is useful for encapsulating and protecting the activity of biomolecules in vivo, but is an arduous process.

Liposome formation has been most commonly carried out by either sonication or extrusion. These methods have been demonstrated to be faster and far simpler, even though this often comes at a cost of lower encapsulation efficiencies. To form liposomes by sonication the lipid mixture is dissolved in chloroform and dried under vacuum at 45 °C for 3 hours. The lipid film is then hydrated in aqueous medium and sonicated for 20 minutes. To form liposomes by extrusion the lipid mixture is dissolved in chloroform, which is removed under reduced pressure. The lipid film is then dispersed in aqueous medium and heated to above the lipid transition temperature. The suspension is passed through a polycarbonate membrane containing specific size pores. Sonication often forms liposomes that were unilamellar or multilamellar, and is less time consuming. Extrusion gives a narrower size distribution than sonication, which is always reproducible.

Phospholipid vesicles are naturally responsive to any stimuli which change the phase of the bilayer from $S_o$ to $I_d$. This change in phase can be achieved using external stimuli such as changes in pH, light and temperature. This sensitivity can be utilised for drug release.

1.2.2 pH sensitive liposomes

A sharp pH change is a known side effect in many pathological problems such as tumour growth and inflammation. pH sensitive liposomes which can release drugs in these specific targets could be of great clinical use. The process of pH destabilisation of the lipid membrane can include; 1) protonating negative lipids in the bilayer which can lead to lamellar/hexagonal phase transition; 2) protonation of negatively charged polymers or proteins embedded in the bilayer which can then
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destabilise the whole structure by lysis, phase separation or pore formation; 3) by acid-catalysed hydrolysis of stabilising lipids into detergents which can destabilise the membrane; 4) ionisation of surfactants into positive and active conjugate acids. Therefore liposomes were created and designed to be passively targeted towards tumour sites. At the target site they underwent membrane disruption and released encapsulated drugs.

pH sensitive liposomes have been used in a range of drug delivery methods. Vyas et al showed that high release rates of doxorubicin occurred in breast cancer tumours. The liposomes were targeted to the breast cancer tumours using estrone, which is site specific for the estrogen receptor. The liposomes fused with the endosome in the tumour cell, as the pH sensitive DOPE could not form a bilayer structure in this environment. This triggered the release of encapsulated doxorubicin. This targeted release gave a greater than six fold increase in the concentration of doxorubicin at the tumour site and significantly decreased the levels of doxorubicin present in the heart, liver and kidney compared to intravenous doxorubicin. pH sensitive liposomes have also been used diagnostically for in vivo imaging. Cardoso et al formed vesicles from DOPE, cholesterol hemisuccinate and DSPE-PEG_{2000}, 5.7:3.8:0.5. These were used to encapsulate 99m Tc-BBN. 99m Tc-BBN is a radiolabelled tetradecapeptide, which has a high affinity for receptors found in tumours, areas such as the lung and prostate were observed. Although higher levels of uptake of imaging liposomes by internal organs such as the spleen occurred, higher levels of 99m Tc-BBN were observed in the tumour than the surrounding tissue. This allowed the tumour structure to be clearly observed while the sensitivity of liposomes to the low pH in the tumour site increased delivery.

pH sensitive liposomes have also been used to regulate intracellular trafficking, gene delivery agents, and cell sensitizers. Among all the reported uses of pH sensitive liposomes it has been demonstrated that they are useful delivery vehicles to tumour and inflammatory sites, where sharp pH changes occur. Although natural targeting is low, it can be achieved through extensive surface modification, utilising proteins, antibodies and synthetic ligands.
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1.2.3 Light sensitive liposomes
Liposomes which are sensitive to light have specific lipids or molecules incorporated into their membranes. Including photosensitive lipids in liposomal membranes has also been used to trigger liposome fusion. These molecules underwent changes such as degradation or isomerisation when irradiated with light at a specific wavelength. The products of this change facilitated membrane disruption. Liposomes formed from DPPC and DC₈,₉PC were photosensitive. Membrane transitions occurred which destabilised the bilayer, releasing encapsulated material, when samples were pulsed with light at a 254 nm wavelength (Figure 1.5). Studies of this phenomenon using calcein showed that a wavelength specific tunable photosensitizer such as calcein was an important part of the lipid destabilisation process. Carrying this methodology through to possible medical applications, doxorubicin was encapsulated in the liposomes which has an excitation/emission spectra of 490/590 nm. Light activation at 254 nm triggered photopolymerisation of DC₈,₉PC and at 514 nm to target doxorubicin, caused membrane destabilisation and the release of the encapsulated drug. This release triggered cell death in a dose dependent manner.

Figure 1.5 a)DC₈,₉PC lipid and b) photosensitive release from membranes doped with lipid.

Photosensitive liposomes can be created by the encapsulation of gold nanoparticles, which are light sensitive. The nanoparticles were embedded in the phospholipid bilayer and absorbed the light energy. This energy was released as thermal energy,
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this temperature increase brought the liposome bilayer above the phase transition temperature which caused release.

Although photosensitive liposomes have been shown to be promising, targeting and release in vivo need to be further optimised. The main hindrance for these systems is the penetration depth that radiation can reach in tissue. Photosensitive release rates are consistently high, with many experiments showing > 80 % release. Obtaining this release in locations in vivo is extremely difficult with the exception of locations such as skin and ocular tissue. This presents a firm barrier which can not easily be overcome.

1.2.4 Thermosensitive liposomes
Thermosensitive liposomes are formed from either thermosensitive lipids such as DMPC\textsuperscript{51} and DPPC\textsuperscript{52} or by the incorporation of thermally sensitive groups such as polymers into the liposome membrane. Liposomes formed from thermally sensitive lipids undergo specific temperature transitions. These occurred as the phase of the bilayer changed as previously discussed in section 1.2.1 (Chapter 1). Liposomes formed from lipids such as DPPC were stable until 41 °C, when the bilayer underwent a thermal transition from gel to liquid crystalline.\textsuperscript{53} This change permeabilised the membrane and released encapsulated material. DPPC is often used in liposomes for biological functions as the DPPC vesicles are stable at physiological temperatures at 37 °C, but only need a small temperature rise to release encapsulated material. This slight temperature change can often occur naturally in pathologically interesting sites such as tumours. This increase has been related to non-neurological vasodilation, increased blood flow and angiogenesis.\textsuperscript{54}

Thermosensitive release of doxorubicin and other cancer drugs from liposome formulations has been studied. Devi et al reported encapsulating methotrexate inside liposomes formed from DPPC:DSPC (9:1).\textsuperscript{55} Methotrexate targets tetrahydrofolate synthesis, which inhibits base pair synthesis and therefore ultimately stops DNA and RNA production. These thermosensitive liposomes with a transition temperature of 41 °C were used to encapsulate methotrexate. These studies showed that the growth
delay on the tumour when liposomal methotrexate was present and thermally released was significantly higher than controls.

Thermosensitive compounds such as acyl chain matched lysolipids were incorporated into lipid membranes formed from DPPC. Doxorubicin was encapsulated into vesicles formed from this mixture to form Thermodox, which showed a sharp release at mild hyperthermia, 39-42 °C. This lipid/polymer mixture is of a higher clinical relevance than the incorporation of other thermally sensitive polymers such as poly (N-isopropylacrylamide). Poly (N-isopropylacrylamide) is not biodegradable and increased levels of the polymer in the body can cause harmful side effects.

Finally, thermosensitive liposomes have often been used in conjunction with hyperthermia generating magnetic nanoparticles, to generate magnetically sensitive vesicles. This increased the targeting and release power of the liposomes. Thermosensitive liposomes can be a useful method for passively targeted release at specific locations in vivo. While this is a useful tool, its selectivity and strength are not high enough for most clinical applications.

1.2.5 Magnetic liposomes

Magnetic liposomes or ‘magnetoliposomes’ are phospholipid vesicles which are closely associated with magnetic nanoparticles. These structures can be formed by; 1) the encapsulation of magnetic nanoparticles into the aqueous core of the liposome; 2) embedding the nanoparticles into the phospholipid bilayer; 3) anchoring the nanoparticles to the outside of the liposome. Recently, magnetoliposomes have also been created by incorporating magnetized polymers into the phospholipid bilayer. When magnetic nanoparticles are used, it could allow the liposomes to be magnetically located to a specific site, e.g. a tumour.

Crucially, magnetic nanoparticles also generate localised heat when placed in an alternating magnetic field. When magnetic nanoparticles were encapsulated in thermosensitive liposomes, this localised heat has been used to trigger release of encapsulated material. Bothun et al reported the formation of DPPC liposomes
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which had small superparamagnetic iron oxide nanoparticles, capped with oleic acid, incorporated into the phospholipid bilayer. Studies showed that the structure was still stable, but the presence of the nanoparticles increased the thermal transition point in a concentration dependent manner. Release studies using carboxyfluorescein showed that release was obtained by using radio frequency heating at 281 kHz.

Magnetoliposomes have also been trialled as clinical diagnostic tools. When used with magnetic resonance imaging (MRI) they can be used to give enhanced images in vivo. Lesieur et al reported encapsulating small Fe$_2$O$_3$ nanocrystals into large unilamellar vesicles formed from egg phosphatidylcholine (EPC) and DSPE-PEG$_{2000}$. The structures caused limited cell toxicity and showed high levels of magnetisation. Blood contrast studies showed that strong negative enhancement was reached easily and maintained for 5 hours. MRI scans showed that organs still had circulating liposomes after 24 hours. Pradhan et al developed a multitargeting system by creating magnetic liposomes which were targeted to the folate receptor. The liposomes encapsulated magnetic nanoparticles and doxorubicin. This allowed them to combine physical targeting using magnetic fields and biological targeting. The liposomes were formed from DPPC:Cholesterol:DSPE-PEG$_{2000}$:DSPE-PEG$_{2000}$-Folate at 80:20:4.5:0.5. They demonstrated triggered release of calcein at 43 °C, and showed high encapsulation levels for doxorubicin, although encapsulation of the nanoparticles was less efficient. When the liposomes were targeted to tumours, the tumour showed a significantly higher uptake of doxorubicin than the approved drug Caelyx™, following magnetically induced hyperthermia.

In 2009 Webb et al reported a novel method of creating magnetoliposomes. Thermosensitive liposomes were formed from DPPC:DMPC:synthetic lipid (Figure 1.6) at a ratio of 85.5:9.5:5. Fe$_3$O$_4$ nanoparticles were formed by coprecipitation, and coated using dopamine tagged histidine. The dopamine chelated to the iron present in the nanoparticles. When the vesicles were mixed with the nanoparticles, large aggregates were formed. Encapsulation studies using fluorescent dyes demonstrated that these aggregates were stable until 37 °C. An alternating magnetic field was also used to trigger release of encapsulated material from the liposomes.
The literature shows that release from liposomes can be achieved in a variety of ways with each method having advantages and disadvantages. High profile areas for targeting *in vivo* such as inflammation and tumours are often associated with thermal transitions and changes in pH. This allows higher release figures of encapsulated material in these areas from thermosensitive and pH sensitive liposomes. These release mechanisms give passively targeted release from thermosensitive and pH sensitive liposomes as conditions for release are only observed in these areas. These biological conditions give thermosensitive liposomes and pH sensitive liposomes an advantage over photosensitive liposomes. Photosensitive liposomes have very specific advantages as light cannot penetrate far into tissue, this forces photosensitive liposomes into very niche applications, such as dermal or ocular drug delivery. However, when photosensitive liposomes are used they often have a higher level of release than thermosensitive or pH sensitive liposomes as physiological conditions show only small variations in temperature and pH.

The most promising delivery vehicles for biomedical applications are magnetic liposomes. These thermally sensitive liposomes can take advantage of any increased temperatures *in vivo*, and can undergo active targeting through the magnetic labels in the liposomes. Magnetic fields can also penetrate deeper into tissue allowing greater reach than light induced release.

### 1.3 Magnetic nanoparticles

Magnetic nanoparticles have been widely used in the literature for applications in biology and medicine. Currently magnetic nanoparticles have great potential in lots of areas of biomedicine such as cell labelling and targeting, tissue repair, drug delivery, MRI and hyperthermia. The nanoparticles need to have high magnetic saturation and biocompatibility.
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Magnetic nanoparticles have a lot of possible uses in clinical applications. They have easily controllable sizes and are in the target size range of cellular uptake. The magnetic nature of nanoparticles is also of great interest as they can be used to tag or label biological entities such as cells, viruses, proteins and genes magnetically.\textsuperscript{70,71} Their magnetic nature also allows them to be manipulated using external magnetic fields. The nanoparticles can act as energy transfer agents. When placed in an alternating magnetic field, the nanoparticles can release the energy absorbed from the field as heat. This ability has led to them being used as thermal agents, delivering hyperthermia to target tissues. The magnetic induction of the nanoparticles is dependent on the strength of the magnetic field, the permeability of free space and the magnetisation.\textsuperscript{33} The magnetic nature of nanoparticles, multidomain, single domain and superparamagnetic depend on the size of the particle.

The most widely used nanoparticles are formed from magnetite and maghemite, as they show good biocompatibility.\textsuperscript{72} Small nanoparticles are used due to their superparamagnetic nature. Superparamagnetic nanoparticles are magnetised when placed in a magnetic field and when the field is removed they relax back to their ground state, releasing energy either through physical rotation of the particle (Brownian motion), or through rotation of the magnetic moment (Nèel relaxation).\textsuperscript{73} The Brownian motion depends on the external environment the nanoparticle is in, whereas the Nèel relaxation depends on the relative energies in the particle of magnetic to thermal.\textsuperscript{74} Superparamagnetic nanoparticles will interact with the field in a frequency dependent manner. Unlike multidomain nanoparticles, superparamagnetic nanoparticles can achieve high levels of hypothermia at low field strengths. This makes them one of the most useful nanoparticles in biological and medicinal processes.

Magnetic nanoparticles are superparamagnetic when the particle size is less than 25 nm.\textsuperscript{75} The domains are so small that they can randomly change direction in the presence of magnetic field or thermal fluctuation.\textsuperscript{76} The properties of the magnetic nanoparticles depend on their size and nanoparticles which are most commonly used have a core diameter of less than 30 nm and therefore are single domain particles.\textsuperscript{77}
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The magnetisation and relaxation of single domain particles is governed by Néel relaxation and Brownian motion.\textsuperscript{76} Néel relaxation is the internal thermal rotation magnetic moment of the particles within the crystal. Brownian motion is the movement of the particle in the solution.\textsuperscript{78} Studies in different solutions can determine which is responsible for magnetisation relaxation and is dependent on the particle composition.\textsuperscript{79}

One of the most useful properties of magnetic nanoparticles, is their reaction in an alternating magnetic fields. The superparamagnetic nanoparticles of iron oxide, have a single domain and when the particles are placed in a magnetic field, the magnetic moment of the nanoparticles continually realigns with the changing field.\textsuperscript{80} An alternating field provides energy which converts magnetic energy to thermal energy, released in the localised area of the particles, with no bulk temperature change. The absorption rate from the alternating field influences the amount of heat generation.\textsuperscript{81} The thermal energy generated by the nanoparticles will vary, depending on the nature of the nanoparticle, the crystal size and the system the nanoparticles are in.

Magnetic nanoparticles are normally made by co-precipitation and then coated for bioapplications. Colloidal synthesis can be used to obtain highly monodispersed nanoparticles.\textsuperscript{82} The technique used to form the nanoparticles has a significant effect on the size of the particles; particles precipitated in the presence of surfactants will have a larger diameter. Surface coatings used on nanoparticles usually fall into four categories; a) nonpolymeric organic stabilisers, such as alkyl phosphonate /phosphate,\textsuperscript{83} b) polymeric stabilisers, such as polystyrene,\textsuperscript{84} c) inorganic molecules, such as silica,\textsuperscript{85} gold,\textsuperscript{86} and gadolinium\textsuperscript{87} and d) targeting ligands, such as albumin\textsuperscript{88} and elastin.\textsuperscript{89} The coating depends on the application the nanoparticles are designed for.

1.3.1 Synthesis of magnetic nanoparticles

Superparamagnetic nanoparticles can be synthesised using a range of processes; electrochemical,\textsuperscript{90} coprecipitation,\textsuperscript{91} hydrothermal,\textsuperscript{92} degradation of iron-organic compounds,\textsuperscript{94} flow injection\textsuperscript{95} and oxidative hydrolysis.\textsuperscript{96} Each method imparts slightly different properties to the nanoparticles.
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Electrochemical synthesis is carried out using sacrificial iron electrodes.\textsuperscript{93} The electrodes are suspended in deionized water at a distance of 2 cm. The current density is varied from 205 to 415 $\mu$A/cm$^2$ for 12 hours. This method gives nanoparticles at ~18 nm diameter with a narrow size distribution.

A novel method for the creation of magnetic nanoparticles is the degradation of iron-organic compounds. This process uses iron acetyl acetonate dissolved in phenyl ether and heated to 200 °C under nitrogen for 120 minutes, then refluxed at 254 °C for 30 minutes.\textsuperscript{94} This gives nanoparticles with a ~5 nm diameter. Larger nanoparticles have been obtained by dissolving iron pentacarbonyl in 1-octadecene and heating 320 °C. This process gave nanoparticles where the size was controlled very carefully, but required large amounts of thermal energy to create the nanoparticles.

Flow injection method for creating magnetic nanoparticles has been used in several different ways. This method uses a similar reaction to flask based co-precipitation. Fe (II) and Fe (III) chloride were dissolved in hydrochloric acid and sodium hydroxide was used as a precipitating agent. Two separate streams were used; one injected the iron-acid solution, the second injected the sodium hydroxide solution. Two methods were used for mixing, the first was continuous flow where the two streams entered the chamber simultaneously.\textsuperscript{95} The second method involved switching the flow so the streams entered the chamber at different times. Each flow set up imparted different properties to the nanoparticles. Continuous flow created nanoparticles of 3 nm +/- 2 nm, while switched flow gave nanoparticles of 5 nm +/- 2 nm. Using this method also showed the effect that reagent concentration had on the nanoparticles. When the concentration of sodium hydroxide was increased, particle size decreased. However, when the concentration of iron increased, particle size increased.

Oxidative hydrolysis is another method of creating magnetic nanoparticles through chemicals and heating. Fe (II) was reacted in DMSO and water under nitrogen.\textsuperscript{96} The mixture was vigorously stirred at 70 °C. This method created nanoparticles with a particle size varying between 4 -25 nm. The size of the particles depended on the water content in the water/DMSO mix. When the water concentration increased, particle size increased. Although this process created nanoparticles easily, it still
used organic solvents which are not well suited for preparation of biomedical materials, and the magnetic hysteresis curves were not strong.

Hydrothermal synthesis is a commonly used technique to create magnetic nanoparticles which are formed from Fe (II) and Fe (III) chloride in hydrochloric acid. The mixture is heated under a neutral gas, such as argon and stirred vigorously, while a basic component such as N(CH₃)₄OH was added. The mixture is then heated to 250 °C for 24 hours. This gives magnetic nanoparticles with an average size of 12 nm. This method has been used to give well controlled nanoparticles without the use of organic solvents but still uses high temperatures.

The most common method for preparing magnetic nanoparticles for biomedical applications is co-precipitation. Co-precipitation uses Fe (II) and Fe (III) chloride in a 1.1 to 2.2 ratio, in hot alkali buffer solutions, formed from hydrogen carbonate and sodium hydroxide. The pH of the reacting solution gave control over the size of the nanoparticles. Altering the reaction conditions during the nanoparticle synthesis such as increasing the pH of the system, decreased the diameter of the nanoparticle. Co-precipitation uses only aqueous solvents and is easily performed at room temperature and pressure. This reaction gives high yields of > 99 % making it suitable for biological and medical applications. It also produces ‘naked’ magnetic nanoparticles which allowed easy coating with any material required for a specific application.

1.3.2 The effect of coating agents
The interactive layer used to cover the nanoparticle will dictate its interaction with the external environment. If hydrophobic groups are used, then the nanoparticles should be easily dispersible in organic solvents. When hydrophilic groups are used, the nanoparticles disperse in aqueous systems. Markovich et al studied the stability that different coatings could give magnetic nanoparticles and how they would affect the nanoparticles physical properties. Their studies showed that the packing density of the surfactants varied. Desorption temperatures and enthalpy binding values suggested that phosphonates and phosphates bound more strongly
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than carboxylates to the nanoparticles. Commonly used coatings include dextran, PEG and aminosilane.

Dextran coated magnetic nanoparticles have been used in several applications, such as MRI contrast imaging. Nanoparticles were synthesised by thermal decomposition of Fe(CO)$_5$. The magnetic nanoparticles were dissolved in toluene and stirred for 12 hours at 25 °C with dimercaptosuccinic acid. The nanoparticles were precipitated, pH adjusted and stirred with triethylamine, EDC, NHS and aminodextran at room temperature for 48 hours. The dextran coatings showed significant enhancement in cellular MRI imaging. Dextran coatings have also provided bioconjugates with enzymes such as urokinase which has been used to target thrombolysis. The dextran coated nanoparticles were synthesised by mixing Fe(III) chloride and dextran in water at 2 °C, then Fe(II) chloride was added. The mixture was stirred under nitrogen while ammonium hydroxide was added and the suspension incubated at 80 °C for 2.5 hours. The resulting precipitation gave ready coated dextran nanoparticles. The addition of the dextran coated nanoparticles to the urokinase the weight of the thrombus was reduced 5 fold.

PEG is routinely used in many biomedical applications to improve biomcompatibility and increase circulation times of delivery vehicles in vivo. PEG has been shown to limit aggregation of magnetic nanoparticles in vivo which is important as large aggregates can have severe side effects in vivo. PEG groups have also been used to coat magnetic nanoparticles, when linked with lactoferrin the PEG coatings made it possible for the nanoparticles to cross the blood brain barrier and deliver drugs to the brain. Fe(acac)$_3$ oleylamine and PEG were stirred under nitrogen for 4 hours at 80 °C. Ether was used to precipitate out the PEG coated nanoparticles. In vivo studies showed that the nanoparticles could be located in the brain.

Aminosilane nanoparticles have been used to target tumour cells. The nanoparticles were formed by co-precipitation and surface modification was achieved by stirring the nanoparticles with 3-aminopropylmethoxysilane in ethanol at 70 °C for 6 hours. The nanoparticles were efficiently endocytosed by lung carcinoma cells and remained present in the cell cytoplasm both pre and post cell division. The nanoparticles were still detected in the cells after several generations. This system
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was studied further in a study of hyperthermia induced tumour reduction. The nanoparticles were injected directly into the prostate and an alternating magnetic field was used to induce hyperthermia. Due to the retention of the nanoparticles, intraprostatic temperatures of upto 48.5 °C were obtained.

As this literature has described, the coating effect of the nanoparticles has been shown to be important in the interactions with the surrounding environment and in biomedical applications. Coatings such as PEG are known to increase circulation times and reduce mononuclear phagocyte system uptake as the \textit{in vivo} systems cannot recognise the polymer. However, as PEG cannot be recognised it equally cannot be used to target specific locations or increase uptake in certain areas.

Dextran and aminosilanes have decreased the circulation time of the nanoparticles in the blood stream but could allow recognition and location of the nanoparticles by the required locations \textit{in vivo}.

1.3.3 Coated nanoparticles for drug delivery

Chen \textit{et al} reported using coated nanoparticles as drug delivery agents for doxorubicin.\textsuperscript{105} Oleate covered Fe\textsubscript{3}O\textsubscript{4} nanoparticles were coated with dopamine, then doxorubicin and human serum albumin. This gave a hierarchacal layered structure with the nanoparticle as the central core. The coated nanoparticles had \textasciitilde50 nm diameter. Release studies showed that full doxorubicin release could be obtained at 37 °C after 50 hours. Nanoparticles without doxorubicin present showed no effect on cell survival. When doxorubicin was present cell growth was significantly inhibited. \textit{In vivo} studies showed the nanoparticles were more successful than free doxorubicin at the tumour site. However, after 24 hours there were higher levels of doxorubicin containing nanoparticles in all organs, particularly the heart and spleen, which were then harder to remove from the body.

1.3.4 Magnetic nanoparticle triggered hyperthermia

As previously discussed it is well documented that superparamagnetic nanoparticles can generate localised heat, when placed in an alternating magnetic field. Cellular injury and or death occur at elevated physiological temperatures for example when
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cellular temperatures rise above 43 °C, cell death was triggered through protein denaturation. This temperature is within the thermal range reached by oscillating field generated hyperthermia in magnetic nanoparticles. This has generated interest in the use of nanoparticles to give targeted cell death in vivo, as this would be an effective anti cancer agent. Couvreur et al reported the use of dextran coated nanoparticles to target solid tumours. Maghemite nanoparticles were coated with dextran and conjugated to folic acid. Folic acid has been used as many cancer cells overexpress the folate receptor, which binds folic acid. The nanoparticles showed steady hyperthermia and low levels of cytotoxicity. Folate targeting was successful, with significantly higher levels of nanoparticle uptake in cells which expressed the folate receptor. Jordan et al reported using 15 nm nanoparticles coated with aminosilane, the formation conditions were not published. In vivo studies in prostate tumours, showed that increasing alternating magnetic field obtained high levels of hyperthermia in the prostate. Temperature levels in the tumour reached 48.5 °C. Nanoparticle stability was maintained in vivo and the nanoparticles were imaged in the prostate 6 weeks after injection. This work demonstrates the possibilities of magnetic nanoparticle triggered hyperthermia as toxic therapeutic agents. The current body of work still does not address the main issues in this area such as, low specificity and increased efficiency which are barriers to clinical approval.

1.3.5 Nanoparticles as intracellular heaters

The use of magnetic nanoparticles in cell targeting and cell localisation and for in vivo imaging has been well documented. For example, Wilhelm et al used magnetic nanoparticles for probing cells and for internal heaters within cells. The magnetic nanoparticles were coated with citrate, to allow dispersion in water and cell internalisation. When the cells internalised the nanoparticles via endocytosis, the nanoparticles were clearly visualised inside the cell via TEM. The cell samples after nanoparticle uptake were then placed in an alternating magnetic field. The magnetic nanoparticles sympathetically aligned their magnetic moment with the magnetic field, which generated localised heating. In the internal volume of the cell, the heating deformed the cell structure and cell membrane, which instigated cell death.
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This method allows small doses of thermal energy to be delivered directly to \textit{in vivo} locations. However, targeting and specificity issues still need to be overcome for this to become a widely used technique.

1.3.6 Magnetic separation using cell-targeting coated magnetic nanoparticles

Liberti \textit{et al} reported targeting tumour cells using magnetic nanoparticles.\textsuperscript{116} The nanoparticles were antibody directed to tumour cells in blood samples. After incubation of the blood samples with these coated magnetic nanoparticles, the solutions were magnetically purified using a permanent magnet, leaving the cancer cells isolated for analysis. Magnetic nanoparticles have also been used in cell counting techniques.\textsuperscript{117,118} Cell location and number were obtained through magnetic measurements either by measuring the cell number in each magnetic scan and averaging over the area, or by measuring the number of magnetic particles per scan and averaging.

1.3.7 Magnetic nanoparticles and \textit{in vivo} imaging

Nanoparticles are playing an increasingly important role in both \textit{in vitro} and \textit{in vivo} diagnostics.\textsuperscript{119} The ability to control the size of the nanoparticles, their biocompatibility, and the ability of these particles to shorten $T_2$ relaxation time of water, makes them ideal diagnostic agents.\textsuperscript{120} Magnetic nanoparticles are normally surface coated to achieve good biodispersity. The nanoparticles can also significantly enhance standard imaging techniques. MRI is one of the most commonly used diagnostic \textit{in vivo} techniques. It uses strong magnetic fields to create 3D images of the \textit{in vivo} environment. MRI is unique as it does not use potentially harmful ionising radiation. Information is gathered by monitoring the magnetic alignment of $^1$H nuclei in the body.\textsuperscript{121} Nuclei at different locations will relax at different speeds. Contrast agents were used to alter the relaxation times $T_1$ and $T_2$ of tissue, giving enhanced images. $T_1$ is the longitudinal relaxation time, $T_2$ is the transverse relaxation time.\textsuperscript{122} Different $T_1$ and $T_2$ will occur in different tissues, allowing enhanced visualisation in target tissue. Many clinical contrast agents shorten $T_1$. Nanoparticles are very useful as contrast agents as they can be used to target $T_2$ relaxation time.\textsuperscript{123} This occurred as the magnetisation of the nuclei was influenced by the spins present on the nanoparticle. The static background magnetisation
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disappeared through phase coherence during measurements leaving only the magnetisation induced by the nanoparticles. When the nanoparticles magnetisation was induced in a perpendicular plane, the difference in magnetisation of the $^1$H nuclei was easily be observed and measured.

Cheon et al reported using magnetic nanoparticles for tumour imaging in breast cancer. The nanoparticles were coated with herceptin antibody, which can target the HER2/neu receptor. This receptor is often overexpressed in breast cancer cells. The herceptin guided the nanoparticles to the cancer cells where imaging occurred using the nanoparticle core. However, when magnetic nanoparticles were used in vivo side effects occurred e.g. coating agents, caused aggregation in the blood stream and disrupted blood flow. Arsalani et al reported the use of PVP grafted magnetic nanoparticles for use in MRI. Unlike previous work where PVP was attached covalently, covalent links between the polymer and the particle gave a much stronger ferrofluid. The synthesised particles displayed good dispersibility and stability in the physiological environment. After covalent functionalisation superparamagnetism was still observed. MRI imaging studies showed considerable contrast enhancement when the nanoparticles were present in a dose dependent manner.

This literature has shown the extensive uses superparamagnetic nanoparticles have in diagnostics. The easily modifiable nature of nanoparticles is a clear asset to their biomedical applications. The breadth of this nanoscale technique has led to improvements in both biological and clinical techniques. The combination of nanoparticles with other established methods such as liposomal drug delivery, increases their reach even further.

1.4 Phospholipid Vesicle Aggregation Methods

The aggregation of vesicles can be very important as most medical applications require vesicles that will not aggregate. Large vesicle aggregates can cause blockages in the circulatory system in vivo which can cause harmful effects. However many biological systems especially in vitro require aggregation. Aggregation can encourage communication between vesicle structures, initiate
vesicle fusion and incorporate other materials into the aggregated structure with the vesicles. The properties of vesicles can also change on aggregation, as they pass from a nanoscale material to a bulk material. Aggregation between vesicles can also allow them to act as simple cell mimics, exhibiting interactions similar to cell-cell contact mediated by proteins such as E- and N-Cadherin.

Vesicle aggregation can occur through many methods, which can be thermally mediated, chemically mediated, polymer mediated, protein mediated and DNA mediated. Some of these methods can lead to fusion of liposomes, others create bulk aggregates formed of individual attached liposomes. Large vesicles have been shown to display longer aggregation times as their increased size effects Brownian motion in solution.

1.4.1 Thermally mediated aggregation
Thermally sensitive vesicles have been studied for their individual release properties. Nonetheless investigations have shown that temperature can also be used to induce aggregation in these vesicles. Levin et al reported that the lipids used to form the phospholipid bilayer can strongly affect whether temperature induced aggregation occurred. Thermal aggregation of vesicles formed from phosphatidylcholine was easier to achieve than phosphatidylserines or phosphatidylethanolamines, due to the surface charges. Liposomes were formed from DMPC, DSPC and DPPC to study aggregation effects. The vesicles were stable at room temperature for 41 days, and changing the phase of the bilayer did not affect aggregation. DSPC and DPPC underwent reversible aggregation rapidly on thermal decreases to 5 °C. However DMPC aggregation was not observed. Torchillin et al reported that temperature dependent aggregation was also observed through increasing temperatures.

Thermally mediated aggregation is a useful in vitro tool. In vivo environments cannot easily undergo induced temperature changes, which made this method highly selective. Although this process is of use by creating aggregation ‘in situ’, reversible aggregation in vivo is largely unachievable, in vivo temperature changes found at target locations such as tumours are not normally extreme enough to effect this type of change.
1.4.2 Chemically mediated aggregation

One of the factors inhibiting vesicle aggregation is the repulsive force between charged phosphate headgroups. Chemicals introduced to a liposome solution can associate with the charged headgroup neutralizing the electrostatic charge and allowing aggregation to occur.

Both monovalent and divalent ions were used by Leonards et al to trigger aggregation.\textsuperscript{133} Using phosphatidylserine and phosphatidylcholine liposomes the aggregation effect of monovalent ions $\text{H}^+$, $\text{Na}^+$, $\text{K}^+$, $\text{Li}^+$ and TMA$^+$ and the divalent ions Mn$^{2+}$, Ba$^{2+}$, Ca$^{2+}$, Sr$^{2+}$ and Mg$^{2+}$ were assessed. When EDTA was added to divalently aggregated liposomes (Mn$^{2+}$, Ba$^{2+}$, Ca$^{2+}$ and Sr$^{2+}$) all showed large reversibility, although some aggregated structures remained, Mg$^{2+}$ showed full reversibility. The effectiveness of divalent ions for inducing aggregation was Mn$^{2+}$ > Ba$^{2+}$ > Ca$^{2+}$ > Sr$^{2+}$ > Mg$^{2+}$ suggesting that the size of the counterion was important. With monovalent ions the effectiveness was Na$^+$ > Li$^+$ > K$^+$ > TMA$^+$, again showing that the size of the ion was important. However, when aggregation was triggered using monovalent ions, full reversibility in all ion samples was obtained in the presence of EDTA. Studies using H$^+$ ions showed that pH dependent aggregation was also possible.

Ion dependent aggregation was also achieved by doping liposome membranes with functional lipids.\textsuperscript{134} Phosphatidylcholine liposome membranes were doped with terpyridine ligands (Figure 1.7).\textsuperscript{135} These showed liposome aggregation in the presence of Fe$^{2+}$, but this aggregation led to fusion and was not reversible. Lehn and co workers also used metal ion induced aggregation by doping EPC liposomes with amphiphilic diketones.\textsuperscript{136} The diketone allowed aggregation of the vesicles in the presence of Eu$^{3+}$. 
Chemically mediated aggregation allows a direct measurable effect in vitro which can be easily controlled. Physiological conditions containing vast arrays of different ions, proteins and biomolecules which all have the potential to interfere with this process, creating barriers to carrying this process forward.

1.4.3 Polymer mediated aggregation
Polymer coatings on liposomes can have many different effects, and a polymer can be chosen for a specific task. PEG has been used to both inhibit and facilitate liposomal aggregation.

The prevention of liposome aggregation is very important in medical applications to prevent blockages in vivo. Choi et al used liposomes formed from DSPC and (p-maleimidphenyl) butynyl-DPPE where PEG groups were attached to lipids covalently to anchor them in the phospholipid bilayer. \(^{137}\) Studies showed that PEG did reduce the aggregation of the liposomes in a weight dependent manner. In contrast Hui et al reported using PEG to induce vesicle aggregation, phosphatidylcholine and phosphatidylethanolamine based vesicles were introduced to PEG\(_{8000}\) in a concentration dependent manner. \(^{138}\) Although the method of aggregation was not known exactly, the concentration of PEG required to stimulate
aggregation was dependent on the lipid composition of the vesicles. Pure phosphatidylcholine vesicles could aggregate in the presence of 4% PEG. As the concentration of phosphatidylethanolamine in the system increased to 75%, a higher level of PEG was required.

Other polymers which have been used to stimulate aggregation are poly(malic acid), \(^{139}\) poly(methacrylic-acid)\(^{140}\) and polyamines.\(^{141}\) These all interacted with liposomes to create aggregates. Biological polymers such as proteins and DNA have also been used to trigger aggregation.

**1.4.4 DNA mediated aggregation**

As with synthetic polymers, the biological polymer DNA interacts strongly with liposomes. This interaction occurs because of the polyanionic nature of DNA and the polycationic nature of liposomes allowing multiple binding sites. The technique of decorating the surface of many liposomes with DNA is an exceptionally useful one. DNA coated liposomes can be used for gene therapy but also for targeted delivery.

There has been interest in DNA-liposome complexes because of their applications as non-viral delivery agents.\(^{142,143,144}\) DNA will interact spontaneously with cationic liposomes, due to its anionic nature, while the ability of DNA to bind to lipids in the zwitterionic state is thought to be mediated through divalent cations.\(^{145,146,147}\) Jeng et al reported using DOPC liposomes to form aggregates in the presence of DNA.\(^{148}\) Only a small amount of DNA ~10 % was electrostatically bound to the liposome surface, but this was enough to induce significant aggregation. The DNA was not observed to intercalate with the phospholipid head groups, binding only to the liposome surface. Studies showed that fusion had not occurred and the aggregation was reversible using NaCl. This system was also studied by Nagayama et al using egg yolk 3-sn-phosphatidylcholine liposomes.\(^{149}\) The liposomes formed ternary complexes with DNA in the presence of cations.\(^{150}\) In this structure DNA partially unwound and semi or full fusion of the liposomes was observed.\(^{151}\) Aggregation of the liposomes was fast, with aggregates formed 60 seconds after mixing. DNA again appeared to be surface bound, imaged between the liposomes using TEM.
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1.4.5 Protein mediated aggregation

If liposomes are to be used in clinical processes it is vital to understand the interactions they will undergo in the presence of biological molecules such as proteins. In clinical uses aggregation is often an unwanted side effect. Studying the effect of proteins on liposomal aggregation can give insights into the behavior of the liposomes in vivo. Tsao et al reported that both trypsin and papain induced liposome aggregation.\textsuperscript{152} Using egg phosphatidylcholine and egg phosphatidylcholine:phosphatidylglycerol mixture liposome aggregation was observed. The studies showed that very low concentrations of trypsin and papain were required for aggregation, and greater aggregation was seen at higher concentrations. Liposomes formed from egg phosphatidylcholine:phosphatidylglycerol were aggregated by both trypsin and papain. Although when trypsin was used disaggregation occurred rapidly, papain aggregation was stable. For phosphatidylcholine vesicles, only trypsin triggered aggregation. The aggregates were disrupted on the addition of salts such as CaCl\textsubscript{2}, MgCl\textsubscript{2} and NaCl. This suggested that the aggregation occurred through electrostatic interactions, similar to DNA. It also suggested that the proteins interact with specific lipids on the bilayer. Liu et al reported annexin II triggered liposome aggregation.\textsuperscript{153} Annexin proteins are specific phospholipid binding proteins and are known to interact with phospholipid bilayers; annexin II is widely distributed in human tissue. Vesicle aggregates were dispersed into the component vesicles upon the addition of dithiothreitol. Liposomes were formed from phosphatidylycerine and phosphatidylycerine:phosphatidylcholine by extrusion. Aggregation occurred quickly. Aggregation could be inhibited through the introduction of N-ethylmaleimide into the system.

Roberti et al reported using a glycoprotein from rat liver endoplasmic reticulum to instigate aggregation, which only occurred at low pH.\textsuperscript{154} Liposomes formed from phosphatidylcholine aggregated in the presence of the protein at pH 5.0. Aggregation of liposomes formed from phosphatidylethanolamine and phosphatic acid were slower. It was thought that the glycoprotein inserted into the membrane before triggering aggregation. After aggregation, fusion of the liposomes occurred making
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the process non-reversible. Fusion occurred due to the enforced close proximity of the liposomes, and fusion helped to reduce spatial and electrostatic strain.

Further studies into exactly how the surfactant proteins, such as trypsin, papain and annexin bond to the lipids present in the bilayer was undertaken by Damodarasamy.\textsuperscript{155} Liposomes were formed from DPPC:phosphatidylcholine:phosphatidylglycerol at a 9:3:2 ratio. Aggregation was induced rapidly on the addition of surfactant protein A, and was dependent on the presence of Ca\textsuperscript{2+} ions. Proteins were produced by mutating the alanine incorporated in the structure. This mutation inhibited aggregation. This work also showed that there are different, regions in the protein which interacted with different chemicals, slight changes to the protein structure inhibited the interactions between the protein and the liposomes, therefore when mutation was used instead of the wild type protein aggregation did not occur.

The literature shows that some proteins can be good, specific triggers for inducing liposome aggregation. Although dependence on the presence of ions and different pH have been observed, this is not always required. Some proteins will react with specific lipids, allowing modifiable systems to cause selective aggregation. One of the most commonly used proteins to induce lipid vesicle aggregation is avidin.

1.4.6 Biotin-avidin mediated aggregation

Avidin is a tetrameric protein which can bind up to four molecules of biotin at any time (Figure 1.8).\textsuperscript{156} Avidin binds biotin through a complex array of intermolecular interactions, including 9 hydrogen bonding interactions and 25 Van der Waals interactions.\textsuperscript{157} Avidin has an exceedingly high binding constant at 10\textsuperscript{-15} M\textsuperscript{-1}.\textsuperscript{158} This interaction is ideal for vesicle aggregation, because biotin tagged liposomes are easily synthesised. Biotin-capped phospholipids are commercially available and avidin is not found in humans, making it a useful in vitro study that will not react in vivo.

Liu et al made an extensive study of biotin-lipid bound interaction with avidin, avidin bound to biotin tagged lipids when they are in a flat bilayer and in liposome.\textsuperscript{159} They reported that the most efficient binding of lipid structures to
avidin occurred when they were in the liposomal form. This gave a theoretical optimum ratio of DPPE and biotin tagged DPPE 20:1. However practical constraints such as sterics and membrane phase reduced the optimum 12:1 ratio. Liu also showed that the inclusion of biotin tagged DPPE did not affect the temperature at which the bilayer underwent thermal phase transition.

Vermette et al showed that the degree of aggregation of the phospholipid vesicles could be controlled. Using DPPC:cholesterol in a ratio of (2:1) with varying amounts of biotin-DSPC-PEG$_{2000}$ it was shown that aggregation was time and concentration dependent, by changing the mole fraction of the biotin lipid. The vesicle aggregates were studied by a variety of techniques to monitor aggregation, including photo correlation and TEM. Studies into the stability of the vesicles showed that the aggregates were stable. This system was further modified by Tilbourg et al to use the liposomes in magnetic resonance imaging.

A similar system is used for clinical applications in vivo. DSPC:cholesterol vesicles with a DSPC:PEG-biotin tag were attached to paramagnetic amphiphiles. TEM studies showed that both biotin tags and paramagnetic amphiphiles did not affect the structure of the liposomes. The liposomes were injected intravenously, and used to image major vessels, such as the vena cava by magnetic resonance imaging. Avidin was then added to the system which induced aggregation, the aggregated liposomes were removed from the body, although the mechanism of clearance was unknown.

Proteins are very useful aggregation tools in liposome chemistry. Avidin due to its high selectivity and binding strength is one of the most utilised. It has been shown to
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be stable under physiological conditions and aggregation using biotin-avidin mediation does not cause liposome fusion and the subsequent release of any encapsulated material in the liposomes.

1.5 Vesicle Gels

Free drugs which are delivered in vivo intravenously are quickly circulated through the body and cleared from the system.\textsuperscript{162} When drugs are encapsulated into liposomes the drug can survive much longer in vivo and can be targeted to specific locations.\textsuperscript{163} However, localising the liposomes into the body can be complicated. Simple ways of protecting the liposome structure for medical application is to support it in a gel matrix.\textsuperscript{164} As these are normally being used for biological and medicinal purposes, a hydrogel is the most suitable. The gel matrix needs to be carefully selected so that it does not penetrate or weaken the structure of the liposomes, and that it is biocompatible and will not trigger an immune response in vivo. The gel can also be used to help target or localise drug delivery. They can also then be used in tissue engineering applications where hydrogels are the chosen material.

\begin{center}
\includegraphics[width=0.3\textwidth]{carbapol.png}
\end{center}

\textbf{Figure 1.9} Structure of Carbopol 974

In 2007 Mourtas \textit{et al} carried out an extensive study of vesicle gels, the effects of vesicle composition, gel viscosity, encapsulation of hydrophobic and hydrophilic molecules and release mechanisms.\textsuperscript{165} The study showed that the lipid composition affected the permeability of the cell membrane and could cause premature release of encapsulated contents. For example, egg phosphatidylcholine showed weaker membrane structure and therefore higher permeability than DSPC and cholesterol. The studies showed that carbapol 974 (Figure 1.9) and cellulose based gels had little effect on the drug release kinetics and the viscosity of the gel did not affect dye diffusion. Mourtas also showed that for release of hydrophilic moieties the rigidity of the membrane was the determining factor of release, whereas release of hydrophobic groups was dependent on drug loading.
1.5.1 Gelatin gels

Gelatin is a naturally occurring polymer formed in most mammals and is composed of amino acids such as glycine and proline (Figure 1.10).\textsuperscript{166} Gelatin is soluble in most polar solvents and can form a thermoresponsive gel. The sol-gel transition temperature of gelatin gels varies between 20 and 30 °C depending on the concentration of gelatin used.\textsuperscript{167} As gelatin is isolated from mammals it has good biocompatibility.\textsuperscript{168} Gelatin does not form a gel under physiological conditions but has been used for localised drug delivery. Gelatin forms gels at room temperature which have been used to support vesicles in medical applications, such as catheter lining and drug delivery, the gel supports vesicle aggregates, and can act as a cell scaffold, or as a coating to allow cells to grow on non-adherent surfaces.\textsuperscript{169,169} DiCosmo \textit{et al} showed that a thin coating of gelatin containing DPPC:cholesterol vesicles formed with ciprofloxacin encapsulated in the aqueous core of the vesicle was stable.\textsuperscript{171} The vesicle/gelatin mixture was used to coat silicone catheters, the catheters were used in clinical environment, their use inhibited bacteria growth for seven days when compared to gelatin coated controls.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.10.png}
\caption{Structure of glycine and proline amino acids}
\end{figure}

Gelatin can also be used to prolong the life of the vesicles \textit{in vivo}. Liposomes can be cleared from the body very rapidly, and even long circulating liposomes (Chapter 1, Section 1.1) can still only be found \textit{in vivo} after twenty four hours by using PEG groups to shield the liposome. In contrast gelatin micro beads have been shown to be stable \textit{in vivo} for up to one month.\textsuperscript{170} Liposomes encapsulated in gelatin should therefore be very useful delivery vehicles for drugs and other medical applications. Gaud \textit{et al} studied the effect gelatin had on vesicle structures.\textsuperscript{171} Liposomes formed from phosphatidylecholine isolated from soybean and cholesterol were held in gelatin gels. Studies showed that encapsulation efficiency in gelatin gels was 83 +/- 2 %. Liposomes coated with gelatin were stable at 70 °C for 40 hours, showing only 1.79
% spontaneous release. In vitro full drug release was achieved at ~25 hours, while in vivo it was achieved at ~30 hours.

These studies show gelatin can stabilise the phospholipid vesicles in vivo, creating novel drug delivery complexes. However, gelatin cannot remain as a gel in vivo due to its thermosensitive properties and so some of its ability to absorb physical stress to protect the liposomes will be hindered.

1.5.2 Chitosan gels

Chitosan is a linear polysaccharide formed from the deacetylation of chitin. Chitosan is formed from D-glucosamine and N-acetyl-D-glucosamine (Figure 1.11). Chitosan has natural antibacterial properties, chitosan forms thermosensitive gels and pH sensitive gels.

[Murthy et al studied chitosan hydrogels as supports for liposome structures. Liposomes were formed from egg yolk phosphatidylcholine and cholesterol at a 60:40 ratio, by the thin film hydration method, giving a range of sizes; the mean size at 220 nm, but ranging from 100 to 500 nm. These vesicle gels were trialled in vivo where liposomes with encapsulated drug showed an extended release profile in the blood plasma than the free drug. This release profile was extended even further by encapsulating the liposomes in the chitosan gel. This showed that the in situ gelling of chitosan gels extended drug circulation times in vivo, it also demonstrated that phospholipid vesicles and chitosan gels were compatible.

Liposomes formed from egg phosphatidylcholine, cholesterol, DMPG and DSPC were used to encapsulate carboxyfluorescein as a model drug. Rheological studies showed that the presence of liposomes did not change the thermosensitivity of the gel process, but as the concentration of liposomes increased the gelation lag
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time decreased. Release studies carried out in vitro showed that encapsulating the carboxyfluorescein inside the liposomes held in a gel matrix, prolonged the release profile. Interestingly the larger the liposomes used to encapsulate the carboxyfluorescein, the lower the level of spontaneous release observed. The liposomes held in the chitosan gel were stable for up to 14 days under physiological conditions, with <20 % of spontaneous release observed. Studies in vitro also showed less encapsulation and release in liposomes formed from egg phosphatidylcholine than phosphatidylcholine and cholesterol liposomes.

Sharma et al reported using chitosan beads to encapsulate liposomes.\textsuperscript{177} Liposomes were formed from phosphatidylcholine, cholesterol and phosphorylethanolamine and used to encapsulate ferric chloride. Release studies showed that spontaneous release from liposomes in the chitosan beads was much slower than spontaneous release than free liposomes with ferric chloride encapsulated. Coating agents on the liposomes appeared to have little effect on the spontaneous release.

1.5.3 Alginate vesicle gels

Alginate is a natural polysaccharide, which is derived from the cell wall of brown algae.\textsuperscript{178} Alginate is formed from $\beta$-D mannanonate and $\alpha$-L-guluronate (Figure 1.12).\textsuperscript{179} Alginates form a variety of structures depending on the composition of the polymer.\textsuperscript{180}

![Structure of alginate crosslinking calcium ions](image)

**Figure 1.12** Structure of alginate crosslinking calcium ions.

Alginates can be composed of only $\beta$-D mannanonate (M) residues or only $\alpha$-L-guluronate (G) residues to give homopolymers. Heteropolymers are formed from either alternating M and G residues such as M-G-M-G-M-G or in blocks such as M-M-M-G-G-G. Each different sequence gives a different alginate structure, but both homo and hetero polymers are anionic.\textsuperscript{181} This allows the alginate to form gels when
mixed with divalent cations. The cations interact electrostatically to give a 3 dimensional gel network. This formation is reversible and the gel can be disrupted if chelating agents such as EDTA are added to it. The EDTA chelates the cations present in the gel matrix, removing the interactions between the strands and the gel network is destroyed. Alginate gels have been controversial as liposome supports. Literature studies are divided into 1) studies which showed the ‘burst effect’ a pattern seen in the release of encapsulated material from the liposomes where release only occurred in a pulsatile manner, 2) studies which showed increased release from the liposomes; the release occurred spontaneously and was enhanced with incubation in alginate, 3) investigations which showed increased release was induced by the divalent ions present and 4) studies which report no increase in release.

The burst effect was reported by Dhoot and Wheatley using liposomes formed from egg phosphatidylcholine, egg phosphatidylglycerol and cholesterol. Studies showed that the ion used to crosslink the alginate had a strong effect on the amount of spontaneous release. Liposomes which were not associated with alginate saw a cumulative release of 30 % over 30 days. When liposomes were encapsulated in alginate crosslinked with Ca$^{2+}$ ions, 100% release was observed at 10 days. When Ca$^{3+}$ ions were used high levels of release were observed by 10 days; however, when Ba$^{2+}$ ions were used to crosslink the alginate gel release was much slower, and full release did not occur in the 30 day period. This was mirrored using liposomes formed from egg phosphatidylcholine, phosphatidylcholine, phosphatidylglycerol and cholesterol. The spontaneous leakage observed was slightly higher, and full release was obtained using barium ions. When the system was studied only using calcium ions to cure the alginate, spontaneous release was shown also to be dependent on the lipids with phosphatidylcholine liposomes showed full release at 12 days, DOPC liposomes at 17 days and DOPC and cholesterol liposomes at 30 days. This demonstrated that this spontaneous ‘burst effect’ must be dependent on both the lipid composition of the membrane and the chemical composition of the alginate.

Langer et al utilised the burst effect nature of liposome-alginate mixtures to deliver drugs. Liposomes formed from phosphatidylcholine encapsulating fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) were incubated in alginate beads.
coated with poly-L-lysine. Two different bursts of FITC-BSA release were observed. The first burst occurred over the first five days, and the second from day 20 to day 40. Unencapsulated liposomes did not show this release. The studies showed that altering the composition of the membrane had a very strong effect on the release pattern.

**Table 1** Lipid composition and alginate permeability of liposomes.

<table>
<thead>
<tr>
<th>Lipids in the bilayer</th>
<th>Bilayer charge</th>
<th>Bilayer state</th>
<th>Burst effect seen</th>
<th>number of days before burst is seen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg Phosphatidylcholine:Cholesterol (1:1)</td>
<td>no charge</td>
<td>Fluid</td>
<td>Yes</td>
<td>20</td>
</tr>
<tr>
<td>DPPC:Cholesterol (1:1)</td>
<td>no charge</td>
<td>Gel</td>
<td>Yes</td>
<td>95</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine:cholesterol:Phosphatidylglycol (4:5:1)</td>
<td>positive charge</td>
<td>Fluid</td>
<td>Yes</td>
<td>13</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine:cholesterol:sterylamine (4:5:1)</td>
<td>negative charge</td>
<td>Fluid</td>
<td>No</td>
<td>not seen</td>
</tr>
</tbody>
</table>

The data in Table 1 shows that having a neutral or positive charge is essential for the pulsatile release behaviour to be observed. It also suggests that the time between the pulses was dependent on the phase the lipid membrane is in, with membranes in fluid phases exhibiting much shorter intervals between pulses than liposomes in the gel phase. The spontaneous release pattern was due to interactions between the liposome and the alginate and the longer the incubation time in alginate solution, the more spontaneous release was observed. This also explained the lack of spontaneous release in negatively charged liposomes, where electrostatic charges repelled the alginate polymers and the membrane was not compromised. In the gel state the membrane is tougher, so the alginate polymer had difficulty penetrating. Studies into the interactions of liposomes with calcium chloride, showed that if the concentration of calcium chloride was below 1 mM, it had no effect on the release of the encapsulated material from the liposomes. It was also shown that leaching of
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calcium from the gels, which caused destabilisation of the bilayer membrane, did not occur. Coating the alginate beads or the liposomes also had no effect. Incubation of liposomes with alginate before crosslinking showed large increases in the sizes of the liposomes, which demonstrated that alginate-membrane penetration did occur, and that this was responsible for the spontaneous leakage.

Literature studies showed that liposome/alginate interactions varied widely as the following studies all reported alginate induced leakage from liposomes encapsulated in the alginate gels. However the leakage displayed was an enhanced spontaneous release profile, not a pulsatile ‘burst effect’ pattern. Li et al studied the release of encapsulated proteins from liposomes in alginate.\textsuperscript{188} Liposomes were formed from egg phosphatidylcholine and cholesterol in a 9:1 ratio and egg phosphatidylcholine, egg phosphatidylglycerol and cholesterol in a 6:3:1 ratio. The liposomes were used to encapsulate bovine serum albumin (BSA) in the core as a model drug. Liposomes were suspended in alginate solutions and crosslinked using Ca\textsuperscript{2+}, Ba\textsuperscript{2+} and Al\textsuperscript{3+} cations. The enveloped rate of BSA was dependent on the cation used, and the lipid composition of the membrane. Phosphatidylcholine, phosphatidyl glycerol and cholesterol liposomes gave a much higher rate than phosphatidylcholine and cholesterol liposomes. Calcium and aluminium cations also gave consistently higher rates of release than barium. Cumulative spontaneous release of BSA from the liposomes was far higher in Ca\textsuperscript{2+} and Al\textsuperscript{3+} crosslinked gels, with full release being obtained in 6 days, whereas barium crosslinked gels showed full release in 24 days in liposomes formed from phosphatidylcholine and cholesterol.

Further studies into Ba\textsuperscript{2+} crosslinked alginate gels showed that phosphatidylcholine, phosphatidyl glycerol and cholesterol were more stable to spontaneous release in gels than phosphatidylcholine and cholesterol. The released BSA was the same as prior to encapsulation, which demonstrated that encapsulation and release did not harm the protein structure. This work did not show the ‘burst effect’ but did show spontaneous release of the protein.

Liposomes were also formed from soybean phosphatidylcholine, and used to encapsulate proteins, before the liposomes were suspended in alginate beads.\textsuperscript{189} The
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studies showed that the release profile of the encapsulated material was pH dependent and dependent on the swelling/degradation of the alginate gel. Higher concentrations of sodium alginate gave higher release profiles. Increasing the concentration of calcium chloride used to crosslink the gel had no apparent effect. Whilst increasing the ratio of liposome/alginate had a significant effect.

Hu et al studied the interactions of liposomes with modified alginate gels. Liposomes were formed from phosphatidylethanolamine and cholesterol in a 4:1 ratio and phosphatidylethanolamine, cholesterol and deoxycholic acid in a 4:1:0.25 ratio. The liposomes were used to encapsulate cis-platin. Alginate was grafted with pluronic F127. The alginate was gelated thermally. The studies showed that the liposomes formed from phosphatidylethanolamine, cholesterol and deoxycholic acid had a larger size, and a higher encapsulation efficiency. The presence of the liposomes in the gel did not have any significant effects on the gel properties. The release studies showed that liposomes in alginate gels showed very low levels of spontaneous release and did not show the burst effect. Liposomes in the alginate gels also showed the highest level of targeted release of cis-platin in vivo.

Bakalis et al studied liposomes which encapsulated both enzymes and alginate inside the liposome. The alginate was encapsulated to protect the enzyme at low pH in vivo. Liposomes were formed from DPPC, and encapsulated alkaline phosphatase or alkaline phosphatase in 1% sodium alginate. Incubation with $\text{Ca}^{2+}$ ions at elevated temperature was used to crosslink the gel in the liposome core. The effect of changing the pH environment of the liposomes was monitored. Liposomes incubated at a low pH changed size over the 10 day time period, but were stable at higher pH. The release of the encapsulated material was obtained by incubation at pH 2, for 2 hours. Release profiles showed that liposomes with alginate encapsulated in the core with alkaline phosphatase showed significantly lower release. Encapsulating the alkaline phosphatase with alginate in the liposome also protected the activity of the alkaline phosphatase. The study did not detail if the burst effect occurred however the evidence presented showed that it was unlikely to.
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Messersmith et al studied the interactions of liposomes with alginate by encapsulation calcium ions and monitoring gelation. Thermosensitive liposomes were formed from DPPC:DMPC at a 9:1 ratio and encapsulated calcium chloride in the aqueous core. Liposomes were also formed encapsulating sodium chloride. These thermosensitive liposomes gave full release when heated to 37 °C. When the liposomes were incubated in alginate solution, this release temperature became lower with increasing concentration of alginate. Liposomes were incubated without alginate at 20 °C for 20 days, the liposomes were then heated to 37 °C and a release profile was observed. However, when the liposomes were incubated in alginate 20 °C for 20 days, then heated to 37 °C no release was observed. The vesicles could also be used to thermally trigger gelation. No burst effect or spontaneous release was seen over a short time scale, as rheometrical studies showed that at 37 °C the alginate became a gel, but at 20 °C it remained fluid.

Investigations into the interactions between the liposomes and the cations present in alginate gels showed that the cations did induce leakage from the liposomes. This system was also studied by Miyake et al, who used L α-phosphatidylcholine to encapsulate calcein as a model drug. The size of the liposomes was variable. Detergent induced calcein release showed significantly higher release than in the presence of calcium chloride or EDTA. These investigations also showed that calcium chloride could be used to induce release of large encapsulation targets such as insulin from liposomes.

Table 2 Comparison of literature data on the burst effect and spontaneous release from alginate vesicle gels.

<table>
<thead>
<tr>
<th>Liposome composition</th>
<th>Modified alginate used</th>
<th>Cross-linking ion</th>
<th>Burst effect observed</th>
<th>Spontaneous release observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg Phosphatidylcholine and cholesterol</td>
<td>no</td>
<td>Ca$^{2+}$</td>
<td>Yes</td>
<td>10 days</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine and cholesterol</td>
<td>no</td>
<td>Al$^{3+}$</td>
<td>Yes</td>
<td>10 days</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine and cholesterol</td>
<td>no</td>
<td>Ba$^{2+}$</td>
<td>Yes</td>
<td>30 days</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Substance</th>
<th>Presence</th>
<th>X (Type)</th>
<th>Reaction</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg Phosphatidylcholine, phosphatidylglycerol and cholesterol</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>Yes</td>
<td>12 days</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine, phosphatidylglycerol and cholesterol</td>
<td>no</td>
<td>Al(^{3+})</td>
<td>No</td>
<td>12 days</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine, phosphatidylglycerol and cholesterol</td>
<td>no</td>
<td>Ba(^{2+})</td>
<td>No</td>
<td>30 days</td>
</tr>
<tr>
<td>Egg phosphatidylcholine</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>12 days</td>
</tr>
<tr>
<td>DMPC, DPPC</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>20 days</td>
</tr>
<tr>
<td>DOPC</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>17 days</td>
</tr>
<tr>
<td>DOPC and cholesterol</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>30 days</td>
</tr>
<tr>
<td>Soybean Phosphatidylcholine</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>8 hours</td>
</tr>
<tr>
<td>Soybean Phosphatidylcholine</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>not known</td>
<td>not known</td>
</tr>
<tr>
<td>DMPC, DPPC</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>DPPC</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>21 days?</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine and cholesterol</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>Yes</td>
<td>6 days</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine and cholesterol</td>
<td>no</td>
<td>Ba(^{2+})</td>
<td>No</td>
<td>15 days</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine and cholesterol</td>
<td>no</td>
<td>Al(^{3+})</td>
<td>Yes</td>
<td>9 days</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine, phosphatidylglycerol and cholesterol</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>Unknown</td>
<td>18 days</td>
</tr>
<tr>
<td>PEA and cholesterol</td>
<td>yes</td>
<td>n/a</td>
<td>No</td>
<td>&gt; 6 hours</td>
</tr>
<tr>
<td>DPPC</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>Unknown</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine and cholesterol</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>Yes</td>
<td>20 days</td>
</tr>
<tr>
<td>DPPC cholesterol</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>yes</td>
<td>95 days</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine, phosphatidylglycerol and cholesterol</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>Yes</td>
<td>Not seen</td>
</tr>
<tr>
<td>Egg Phosphatidylcholine, sterylamine and cholesterol</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>13 days</td>
</tr>
<tr>
<td>DMPC, DPPC</td>
<td>no</td>
<td>Ca(^{2+})</td>
<td>No</td>
<td>20 days</td>
</tr>
</tbody>
</table>
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The data in table 2 shows that the interactions between the alginate gel and the phospholipid liposomes are very complex. It is challenging to decide the exact cause of the burst effect and in which systems it is likely to occur. The collated data (table 2) shows that the burst effect is more likely to be seen when using crosslinking ions such as calcium as opposed to barium. The data also suggests that liposomes formed from DPPC are less likely to demonstrate the burst effect than egg phosphatidylcholine. Liposomes containing cholesterol often demonstrate the burst effect, but it is not a guaranteed observation. All the liposomes demonstrate some spontaneous release, however full release might only be reached after hours to days. The time it takes to obtain full release from the liposomes does not appear to be reliant on the lipid, the crosslinking ion or the alginate. Full spontaneous release was reported for liposomes formed from phosphatidylcholine at both 8 hours and after 12 days. Both of these data sets were in alginate crosslinked with calcium ions. Phosphatidylcholine and cholesterol liposomes are also reported to show full release at 10 days, 12 days and 30 days. This shows that the ‘burst effect’ demonstrated by liposomes in alginate is not an easily reproducible experimental observation, but depends on a complex set of interactions in a multicomponent system and is reliant on many different factors.

Liposomes have also been used as a template to create nanoparticle sized alginate gels. Raghavan et al formed liposomes from DPPC, cholesterol and DCP (Figure 1.13). DCP prevented aggregation of the liposome as its negative charge caused electrostatic repulsion. The liposomes encapsulated 1% wt/vol of sodium alginate in the aqueous core. The liposomes were incubated in calcium chloride, which diffused through the membrane in its permeable phase (at higher temperatures), crosslinking the alginate which produced the gel inside. Detergent was then used to disrupt and disperse the liposome membrane, leaving an alginate nanoparticle. The nanogels had a slightly larger size, (~75 nm), than the liposomes at (~65 nm) which was due to the swelling of alginate gels in aqueous solutions. The nanogels were eluted to separate them from the formation environment and monitored using light scattering; only gelled nanoparticles showed high intensity light scattering, the template liposomes and ungelled alginate showed 8-fold less intense scattering. The size of
the nanogels was dependent on the salt concentration, Na\textsuperscript{+} replaced Ca\textsuperscript{2+} in the matrix which loosen the structure and swelling the particles; this demonstrated that the properties of the gels and their response to the external environment was the same nanoscale as in bulk.

This method of creating nanogels was utilised by Monshiporari \textit{et al} to create novel drug carriers.\textsuperscript{195} Liposomes were formed from DPPC, giving multilamellar liposomes, which were then extruded to give large single lamellar liposomes. Sodium alginate solution was encapsulated in the aqueous core of the liposome. The liposomes were incubated with calcium chloride to gel the alginate inside the liposome core. Cytochrome-C was also encapsulated in the liposome core as a model drug, while aggregation of the liposomes was prevented using sodium citrate. The alginate nanogels were freed from the liposomal coating using octyl β-glucopyranoside (OBG) detergent. Liposomes containing gelled alginate nanoparticles were larger in size than the liberated alginate nanogels, in contrast to the size difference reported by Raghavan \textit{et al}. The presence of sodium alginate in the vesicle core did not affect the properties of the phospholipid bilayer. However, when the alginate was crosslinked with Ca\textsuperscript{2+} ions the phase transition temperatures increased. Release studies showed that when release of cytochrome-C was monitored, alginate nanogels and liposomes with alginate encapsulated already showed 50 % release at time=0. Liposomes which encapsulated cytochrome-C only did not show this burst effect release. Full release was not achieved from any of the systems after 45 hours; alginate nanogels showed 80 % release, alginate nanogels encapsulated in liposomes 60 % release and liposomes with no alginate present 40 % release.
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Although alginate gels create very complex behaviour as vesicle supports, they have still been used extensively. The controversies over whether they can trigger spontaneous release from liposomes, and the exact mechanisms by which this occurs are offset against the advantages of cost, biocompatibility, fast gelation and the ease of gel formation/degradation. Literature studies have shown that they can be used with liposomes as structural supports, to complement their drug delivery behaviour and to create novel delivery vehicles. The burst effect which has been observed appears to be dependent on the incubation period, and not dependent on the lipid choice. This means it can be minimised, and allow alginate to be a suitable hydrogel support for liposomes.

1.6 Liposomes as Cell Mimics
Phospholipids formed into vesicles are extremely useful for in vitro studies as they can model aspects of how cells function. Cells all have a phospholipid bilayer as a biological membrane which contains the cell and separates it from its external environment. The cell membrane is formed from phospholipid bilayer with proteins embedded in it and decorated with carbohydrates, glycoproteins and glycolipids on the external surface. This phospholipid bilayer contains an average 500-1,000 different lipid species. According to the fluid mosaic model the cell membrane is classed as a two dimensional liquid which allows the diffusion of lipid and protein structures. Protein structures which are embedded into the phospholipid bilayer often have a very specific structure consisting of hydrophilic groups at each end and a hydrophobic section which spans the membrane. The lipid bilayer is the matrix of the ‘mosaic’. The bilayer undergoes many interactive forces; however, the force which holds it together is the hydrophobic effect. The bilayer does not exhibit long range order due to its fluid nature, but can exhibit short range order as specific molecules interact with each other. The lipids do move around inside the layer, such as the flip-flop motion, where the membrane is fluid enough to allow a lipid on the external surface of the membrane to traverse the bilayer to the inner surface (Figure 1.14). Lipids of a specific type can aggregate together to form a lipid raft. These lipid microdomains are believed to play key roles in signalling. The lipid raft can also move freely in the bilayer.
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Figure 1.14 Flip flop effect showing the transition of lipids between the external and internal areas of the bilayer.206

Studying liposomes as cell mimics is very important as it allows the transport of compounds in and out of cells to be understood. Transport across cell membrane occurs via ion channels, ion carriers or endocytosis. Transport across liposome membranes can be achieved by the same methods. Ion carriers facilitate the diffusion of the substrate (normally an ion) across the membrane. Substrates are brought across the membrane by forming a non-covalent bond with the ion carrier, the ion carrier then diffuses through the membrane, the substrate is released and the ion carrier diffuses back across the membrane.207 Ion carriers often have a macrocyclic structure which provides multiple anchoring points for the substrate. Natural ion carriers included antibiotics such as valinomycin which selectively transported ammonium ions.208 Other ion carriers which have been isolated include monensin which transported positively charged ions, but under physiological conditions showed selectivity for sodium ions.209 Synthetic structures used as ion carriers often mimic nature and are based on crown ethers and cryptands.224

Ion channels differ from ion carriers as they remain constant and span the width of the membrane. They normally are formed from tubular or helical structures such as peptides which allow them to act as pores or channels through the membrane.210 Naturally occurring ion channels are often based on the structure of toxins or antibiotics such as gramicidin A.211 Gramicidin A is a linear peptide which complexes to univalent cations, transport across the membrane is ion specific with sodium ions transported sequentially but multiple caesium ions can be transported simultaneously.212

Literature studies have focussed on synthetic ion channels, such as those reported by Lehn et al, who constructed dendritic molecules which closely mimicked the action
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of protein channels in vivo. Liposomes were formed from phosphatidylcholine, and incorporated the complex dendritic structures into the phospholipid bilayer (Figure 1.15). The liposomes encapsulated Li\(^+\) ions and were suspended in a solution of Na\(^+\) ions. The studies showed that the entry of Na\(^+\) ions into the liposomes was entirely dependent on the presence of the channel structures. Simpler structure shown in figure 1.15b showed an increased level of Na\(^+\) ion uptake than the more complex structure shown in Figure 1.15a. The amount of Na\(^+\) ions taken up into the liposome core was of a similar concentration to the Li\(^+\) ions displaced and removed from the liposome core. This work has shown that liposomes and synthetic ligands have mediated ion transport in a cation-cation transfer method.

![Figure 1.15 Lehn dendritic membrane spanning structures a) complex structure and b) simple structure.](image)

Gräber et al reported the transport of protons across liposome membranes. Liposomes were formed from phosphatidylcholine and phosphatidic acid. The pH of the liposome core was maintained at pH 6.40 and the pH of the external solution was maintained at 8:45. The liposomes had F type H\(^+\)-ATPases embedded in the membrane, which were responsible for proton transport. The transport of protons catalysed the ATP→ADP reaction. The transport occurred in a rapid manner.
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One of the most interesting methods of transport across lipid membranes is by inducing a phase change in the membrane, which destabilises the membrane releasing the contents. As the membrane changes phase the lipid molecules in the bilayer rearrange from a highly ordered tightly packed arrangement in the gel state, to a loosely arranged order in the liquid crystalline state, diffusion across the membrane can occur easily.\textsuperscript{215} This phase transition is easily effected in thermally sensitive liposomes such as DMPC and DPPC by raising the temperature to trigger release. This has been used for drug delivery for a range of compounds including doxorubicin and methotrexate as previously discussed (Chapter 1, Section 1.2.4).

Transport across the membrane is a diverse problem to which many solutions have been tried. A simple method of cross bilayer transport is to effect a disruption in the bilayer which will allow diffusion of encapsulated material out of the liposome, and this has been used extensively though it lacks the specificity that ion carriers and channels can give rise to. When selecting a method for transportation the questions that arise centre on if the use of external stimuli are possible and if high levels of selectivity are required. In most \textit{in vitro} experiments and \textit{in vivo} drug delivery specificity is not required as the target is simply to deliver the encapsulated material and stimuli such as heat can be easily applied to the system.

1.7 Tissue Engineering

Tissue engineering is the process of creating tissues from cells and materials \textit{in vitro} which can carry out biological functions, and replace lost or damaged tissues \textit{in vivo}.\textsuperscript{216} The material used to support cell growth and act as a biological matrix is very important, and needs to be selected carefully. The matrix needs to mimic the \textit{in vivo} three dimensional environment closely.\textsuperscript{217} Materials used need to be biocompatible and be recognised by the cells, allowing cell attachment and migration of other cells into the synthetic tissue.\textsuperscript{218} The material also needs to be porous to allow diffusion of nutrients and oxygen to the cells inside its matrix.\textsuperscript{219} The physical properties such as stiffness and strength of the material also play important factors with cells such as chondrocytes growing better on stiffer matrices similar to cartilage, and cells such as myoblasts, preferring softer matrices similar to muscle.\textsuperscript{220,221} Materials used in tissue engineering are varied and include bioactive
glass, electrospun fibres, textiles such as silk, polymers, thin films and metals. Hydrogels have been greatly researched for tissue engineering purposes, as some of their characteristics closely mimic the extracellular matrix and they have great potential for tissue engineering and 3D cell culture.

The hydrogel used is highly dependent on the final purpose of the engineered construct. Hydrogels used to mimic bone are formed from polyethylene glycol and polylactic acid. Cardiovascular implants often use fibrin, polyethylene glycol, hyaluronic acid and alginate. Hydrogels supporting chondrocytes to mimic cartilage use polyethylene glycol, alginate, collagen, fibrin and hyaluronic acid. Vascular engineered constructs use polyethylene glycol and hyaluronic acid.

The range of hydrogels which have been utilised for tissue engineering is vast. The most relevant hydrogels utilised in biomedical applications to this work are gelatin, chitosan, polyethyleneimine, collagen, polyethylene glycol, chitosan: polyethyleneimine and alginate.

The purpose of hydrogels is to mimic the natural environment of the cells in vivo as closely as possible. Using extracellular matrix components has an advantage as they are easily recognised by the cells, and therefore have high levels of cell attachment and biocompatibility. Unfortunately, these gels are not always easy to obtain or handle, and high levels of purification are needed for biomedical applications. Hydrogels which can act as cell scaffolds can be synthesised with relative ease. These gels may not be recognised by the cells but can be designed for specific gelation properties and can be produced at high levels of purity. This study will look at both the natural and synthetic types of cell scaffolds which can be used, each with their own advantages.

1.7.1 Natural gels as cell scaffolds

1.7.1.1 Collagen

Collagen is a protein which is abundant in mammals. Collagen is often located in connective tissue and is found in high levels in extracellular matrix. Collagen is
formed from many amino acids and exists in many different forms. Each type of collagen is found at a different site \textit{in vivo} and each has slightly different properties. As collagen is a natural component of many extracellular matrices, it is easily used as a cell scaffold \textit{in vitro}.

Gibson \textit{et al} studied the structure of collagen based gels and the effect of structure on adhesion of mouse osteogenic cells.\textsuperscript{236} Type I collagen gels were modified slightly by the addition of chondroitin-6-sulfate. These gels were formed easily by freeze drying, which encouraged co-precipitation and gave a highly porous structure. The freeze drying temperature had a strong effect on gel formation with pores of 150 \textmu m observed at -10 °C and pores of 90 \textmu m observed at -40 °C. The pore sizes present in the collagen gel network altered the attachment profile of the cells. Smaller pore sizes of 95 \textmu m showed ~50 \% cell attachment. The extent of attachment decreased as pore sizes increased. Gels with an average pore size of ~150 \textmu m only showed 20 \% attachment. The cell attachment did not increase with incubation time, but at small pore size cell proliferation was observed.

Miyata \textit{et al} studied the effect of pure collagen gels on cell growth.\textsuperscript{237} Collagen gels were formed from collagen types I, II and III. The collagen gels were dissolved under acidic conditions and then neutralised using ammonia gas to obtain a gel. The gel was then freeze dried and used for cell culture. The gels were used as cell scaffolds for human fibroblasts, bovine endothelial cells and hamster fibroblasts cells. Proliferation studies showed that all the cells adhered to and proliferated on the collagen scaffolds, but fibroblast cells showed a faster rate of proliferation than endothelial cells. SEM images showed that the cells adhered to the structure well.

Collagen II is a major component of cartilage, and is produced and excreted by chondrocyte cells.\textsuperscript{238} Various types of collagen have been used as a scaffold with chondrocyte cells for use as a bioimplant. The collagen gel was formed from porcine type I and type III collagen.\textsuperscript{239} Chondrocytes were isolated from bovine nasal septum fragments. Histological studies showed that matrix formation occurred around the implant \textit{in vivo}, but this process was dependent on the chondrocytes present in the scaffold. Cell migration from endogenous chondrocytes was observed.
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By labelling the implanting cells, microscopy clearly showed that cells migrated from the implant into the surrounding cartilage. The tensile strength of the implants was higher in the presence of chondrocytes, and studies showed that integration levels of the construct into the cartilage were high.

The interactions of chondrocytes with collagen scaffolds are not identical and vary greatly between different types of chondrocytes and their interactions with different types of collagen scaffolds. Zhang et al studied the auricular, articular and meniscal chondrocyte interactions with type II collagen and type I/III collagen scaffolds. Incubation of the collagen I/III with cells showed decreases in the structure of the collagen, where auricular chondrocytes contributed to the highest levels of degradation, with a 20% decrease in structure diameter. However, in type II collagen meniscal chondrocytes contributed far higher to the degradation of the collagen, with a 40% decrease in structure diameter. The extracellular matrix formed by the chondrocytes varied greatly. Auricular chondrocytes produced high levels of elastin, while smooth muscle actin was produced by meniscal chondrocytes. Lubricin was formed in low levels by articular chondrocytes whereas it was observed in higher quantities in meniscal chondrocytes. The production of extracellular matrix components by the chondrocytes was affected by the differing collagen gels. Auricular chondrocytes incubated in type I/III collagen showed lower levels of collagen II production. However, when incubated in type II collagen, the production of collagen I was not significantly diminished. Full studies of the extracellular matrix of all cells in both collagen matrices showed that by changing the scaffold, from collagen type I/III to collagen type II, only showed an effect on the cell density of each sample, and the production of collagen I and II.

Collagen is an easy to obtain biopolymer but as this work shows, its formation as a cell support cannot be obtained under physiological conditions and its interaction with cells varies and is hard to predict. However, its many advantageous properties include high levels of cell adherence, good biocompatibility and high levels of cell proliferation.
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1.7.1.2 Chitosan
Chitosan is a polysaccharide which is formed from N-acetyl-D-glucosamine and β-(1-4)-linked D glucosamine. Chitosan comes from the exoskeleton of crustaceans in the form of chitin, which is then deacetylated to give chitosan. Chitosan has been used in several bioapplications due to its antibacterial properties and charged nature. Due to its polyionic nature, chitosan gels are often formed through pH changes as previously discussed in section 1.5.3 (Chapter 1).

Gilbert et al report using chitosan as a cell scaffold for chondrocytes isolated from pigs. Cells adhered well to the chitosan scaffold and appeared to be live and viable. Histological staining for collagen II showed that the cells had begun to deposit extracellular matrix. This showed that chondrocyte cells could interact well with chitosan.

Lee et al used chitosan gels which formed in situ, where the linear polysaccharide chains of chitosan were crosslinked with glycerol phosphate. The phosphate anions of the glycerol phosphate interacted electrostatically with the cations that chitosan displayed. The amount of glycerol phosphate present in the chitosan gels, affected the physical properties of the gel, making the thermal stability of the gels variable. When no glycerol phosphate was present the gels showed no change in viscosity up to 60 °C. As the level of glycerol phosphate was increased, the thermal related viscosity of the gels decreased. This allowed the mixture containing 20 % glycerol phosphate to be fluid at 20 °C but it formed a gel at 37 °C, meaning that gelation was achieved upon injection in vivo. SEM showed that the cells attached well to the chitosan gels. Viability studies showed that the cells did proliferate on the chitosan gels over a 7 day period, but at a slightly lower rate than on culture plates. Studies after implantation in vivo showed that the chitosan did not disperse, although cell migration was seen. Some vascularisation was observed, but immunofluorescence showed that the implanted cells did not migrate from the chitosan gel. Some immune response could be detected in vivo after 28 day implantation, but it was at a low level and appeared to be concentrated in tissue rather than chitosan gel.
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Ide et al used chitosan gels as scaffolds for bone marrow stromal cell derived Schwann cells. Chitosan gels were formed by dissolving chitosan in acetic acid. Upon neutralisation gelation occurred. Bone marrow stromal cells were incubated under differential conditions to obtain Schwann cells. Immunofluorescence using P75 and PO which are markers for Schwann cells, showed differentiation had occurred. Chitosan gels with Schwann cells encapsulated, were implanted into gaps in the sciatic nerve. Immunostaining after 7 days showed that regenerating axons extended into the chitosan gel. The transplanted cells were located in the regenerating axons and in the stump at the gel/nerve interface. These effects magnified and increased at 14 and 28 days post implantation. Further studies after 2 and 4 months of surgery showed well myelinated axons through the gel structure in both the tibial nerve and the peroneal nerve.

Buschmann et al used chitosan to deliver cells in vivo. Chitosan based gels were formed by mixing acid dissolved chitosan with glycerol phosphate and glucosamine. The mixtures were crosslinked with hydroxyethyl cellulose to form gels. Chondrocytes were isolated from calf cartilage. Live/dead assays showed that cells were viable and proliferating. Studies into RNA and protein expression both in vitro and in vivo revealed that expression levels were higher after longer incubation periods in vitro due to increased cell proliferation. This effect was not observed in vivo. Some changes in expression were observed between the in vitro and in vivo samples. This was seen particularly in collagen production. In vitro collagen type II was strongly expressed in agarose gels containing chondrocytes and cartilage, but was barely seen in chitosan both in vitro and in vivo implants. Cell density in chitosan implants increased over time as did glycosaminoglycan production. This work shows that the chitosan gels can be used to deliver cells in vivo using in situ gelling, although the properties of both the cell and the gel may change.

1.7.2 Synthetic cell scaffolds

1.7.2.1 Polyethyleneimine

Polyethyleneimines (PEI) are polymers formed from saturated carbon chains linked by amine groups (Figure 1.16). PEI gels are formed in water, and are often used as a modification of other gel structures to promote cell attachment. PEI is polycationic
which attracts the negative charges often found on the surfaces of cells.\textsuperscript{247} Also due to its cationic nature PEI can be used successfully as a transfection agent.\textsuperscript{248} Polyethylenimine in high concentrations can trigger cytotoxicity and is therefore rarely used alone.\textsuperscript{249}

\begin{center}
\includegraphics[width=0.5\textwidth]{figure16.png}
\end{center}

**Figure 1.16** Structure of PEI

PEI has been used mixed with other hydrogels, such as carboxymethyl cellulose and chitosan. Kim \textit{et al} used PEI gels mixed with carboxymethyl cellulose, in an \textit{in vivo} experiment to test cytotoxicity and immunogenicity.\textsuperscript{250} The gels were formed from PEI 76 % wt/vol, and carboxymethyl cellulose 3 % wt/vol. Gelation was thermally triggered. The mixture was fluid at room temperature and gelled at 37 °C, which is of course of use in biological applications as it allows \textit{in situ} gelling. The mixture was an easy to handle fluid prior to injection \textit{in vivo} where it becomes a gel. The gel matrices were also used as slow release stores of drugs or proteins, which was modelled by the release of bovine serum albumin (BSA). The solution of BSA was added to the gel prior to gelation, and released slowly over a 10 day period, when full release was obtained \textit{in vitro}. \textit{In vivo} studies show that release of BSA occured over 16 days – measured using the concentration of FITC-BSA present in plasma. Histological sections showed that the gels were infiltrated easily by cells such as macrophages, neutrophils and lymphocytes. Immunostaining demonstrated that large numbers of cells were present through the gel structure, with a few macrophages present mostly located at the edge of the implant.

PEI is paired with chitosan to form mixed gels. Chitosan is a polyionic biopolymer which is used extensively due to its biocompatibility, as previously discussed. Polyethylenimine has a major use in gene delivery bioapplications. Bradley \textit{et al} studied a mixture of these two polymers as a possible cell scaffold.\textsuperscript{251} The hydrogels were formed by mixing chitosan 2 % in acetic acid, with PEI 10 % in water; Gelation occurred at 25 °C after mixing. The structure of the hydrogels was dependent upon the ratio of chitosan to PEI; at a 10:90 ratio, clear gels were formed, that were stable. At a ratio of chitosan: PEI of 40:60 white gels which were tough
were formed. Rheological studies showed that the gels degraded over a 28 day period under physiological conditions to half their compressive modulus and they lost ~50% of the gel structure. Thermal and pH swelling experiments showed that the gels were best supported at a higher pH, where they retained more water in the gel structure (up to 40 °C). The cell scaffolds were implanted with HeLa cells. Studies at 21 days showed the cells were present and viable in the gel and further immunostaining experiments showed cell proliferation. Gels were used as scaffolds for human fetal skeletal cells which showed increasing levels of Pcna expression (a marker for cell proliferation). Further studies showed that the gel promoted cell proliferation and hindered dedifferation in comparison with standard in vitro culture.

A slightly modified version of the chitosan:PEI mixture was used by Ku et al. to regenerate cartilaginous tissue. The gels were formed from PEO(polyethyleneglycol), chitosan and chitin, in a 20:25:55 ratio and then modified with PEI to include 390 µg/mL of PEI in the gel. The gels were crosslinked using genipin (Figure 1.17). The presence of the PEI in the gel throughout the structure was confirmed by FITR spectroscopy, where peak transmittance was at 1010 cm\(^{-1}\) when no PEI is present, it changed to 790 cm\(^{-1}\) when PEI was incorporated into the structure. The cells were seeded with chondrocytes isolated from bovine knee. The concentration and the location of the PEI in the gel had no effect on cytotoxicity. When the concentration of PEI in the gel was increased from 0 to 390 µg/mL in the bulk gel structure, the percentage viability of the cells dropped from 96% to 94%.

Figure 1.17 Structure of Genipin

However when the concentration of PEI on the surface increased from 0 to 390 µg/mL, the viability dropped to 89%. This demonstrated that the PEI was more toxic on the surface, probably as it formed localised dense areas of polymer. However, cell adhesion studies showed that a much higher level of cell attachment as observed when PEI was only present on the gel surface. Cells grown on surface modified gels also deposited more GAGs and collagen.
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*Table 3* Table comparing literature data for hydrogels as cell scaffolds.

<table>
<thead>
<tr>
<th>Gel</th>
<th>Cell type</th>
<th>Reference</th>
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<tr>
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<tr>
<td></td>
<td>Chondrocyte</td>
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<tr>
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<td>mesenchymal stem cell</td>
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<td>calvaria preosteoblast</td>
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<tr>
<td></td>
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<tr>
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<td>smooth muscle cell</td>
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<tr>
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<td>Fibroblast</td>
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<tr>
<td></td>
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As Table 3 shows each hydrogel can be used for a range of cell types. As cells adhere and proliferate on these surfaces other factors need to be taken into consideration when selecting a hydrogel for a biomaterial, such as the immunogenicity of the material, the cost and the difficulties in handling, storing and triggering gelation of the hydrogel. The variety of hydrogels used in tissue engineering is extensive, and each hydrogel has many advantages and disadvantages. A hydrogel which has been studied as a cell scaffold due to its ability to trigger no immune response and its quick easy gelation process is alginate. It has been trialled in a range of tissue engineering applications and has only one major disadvantage which is poor cell adherence. However this has been overcome in many applications through supramolecular or synthetic modification.

1.7.3 Alginate gels
Alginate is a useful material in tissue engineering. It is a fluid solution under physiological conditions, and can be crosslinked using mild, biocompatible chemical such as Ca$^{2+}$ ions (Chapter 1, Section 1.5.7). The crosslinking process is reversible by the addition of chelating agents such as EDTA, which are also mild, and to some extent biocompatible. As alginate is isolated from algae it is not recognised by mammalian tissue, and therefore elicits a low immune response when used in vivo.\(^{267}\) The major drawback to using alginate as a cell scaffold is the lack of recognition by cells, due to its algae origins. Mammalian cells find it hard to adhere to the alginate structure, but attachment can be achieved through covalently bonding recognisable motifs onto the alginate surface such as the peptide sequence RGD.\(^{268}\) It is also possible to incorporate proteins from extracellular matrix such as fibronectin into the gel structure, to increase adhesion by the cells.\(^{269}\) In some cases cells such as chondrocytes, which have a preference for a more rounded less adherent morphology, that more closely mimics their environment in vivo, increasing attachment is not necessary and only encapsulation in the gel matrix is required.\(^{270}\) Barralet et al used alginate as a cell scaffold for rat bone marrow cells.\(^{271}\) Gels were formed from alginate containing different quantities of guluronic and mannuronic residues. Gelation was achieved by the addition of calcium chloride. Incubation under physiological conditions caused degradation of the gel structures, with alginates containing a higher level of mannuronic, stability was good initially, but
then degraded faster than alginates containing a higher level of guluronic. The composition of the alginates had a strong effect on the proliferation of cells in the alginate matrix. Alginates which contained a high level of guluronic acid showed good proliferation rates. However alginates which had a high level of mannuronic acid and reagent grade alginate (gluronic acid : mannuronic acid 60:40) both showed little cell proliferation. Microscopy showed that the cells could adhere and grow on the gel showing spread, adherent morphologies.

Alginate modified with the peptide RGD was used to regulate cell behaviour by controlling stiffness of the gel. Alginate was modified by altering the secondary alcohol groups to methacrylate groups. The methacrylate groups were photosensitive, the alginate was also covalently modified to attach RGD to the polymer backbone (Figure 1.18). 3T3-L1 cells were cultured on the scaffolds and gelation was triggered using photoinitiators, Igracure 2959 and VA 086. Igracure 2959 showed high levels of cytotoxicity. A 15 minute incubation period resulted in total cell death. By varying the concentration of the photoinitiator the stiffness of the gel could be controlled. The cells interacted more strongly with RGD modified alginate with the cell area covered, and the average cell number showing a 2 fold increase. The effect of the stiffness of the gel on the cell was also seen, as a higher cell number was observed on stiff and moderate gels and a greater proportion of the cells were viable, in comparison to softer gels. Differentiation of the cells was far higher in the softer gels than the stiff gels.

Modified alginate gels were used by Eslaminejad et al to repair cartilage defects. Alginate polymers were covalently modified to include octadecyl chains onto the alginate backbone (Figure 1.19). The gels were cured using calcium chloride. The alginate gels were used to culture primary bone marrow cells and mesenchymal stem cells. Microscopy showed that the cells adhered well to the scaffold. Cell proliferation studies showed that the cell number in the scaffolds was stable and proliferation was not observed.
Real time-PCR showed that the cells still showed collagen II, Sox 9, aggrecan and GADPH at 14 and 21 days in culture, comparable to controls.

Mow et al investigated the effect of calcium chloride concentration on alginate-chondrocyte structures. Most in vitro experiments use low level concentrations of calcium ions, between 1 and 2 mM. However, some target sites in vivo display far
higher levels of calcium particularly in cartilage where concentrations of calcium can reach 4 mM. Alginate gels were formed using 50 mM of calcium chloride and the samples were then incubated at 1, 1.8 and 4 mM calcium solutions. The studies showed that the shear modulus of the gel increased with increasing concentrations of calcium ions, as did the compressive modulus. When the gels were seeded with chondrocytes from calf cartilage the concentration of calcium ions was found to affect the cells. As the concentration of calcium ions increased, the production of deposited collagen and glycosaminoglycan decreased. The amount of DNA present in each sample also decreased as the concentration of calcium ions increased, suggesting that fewer cells are present in the gel at higher concentrations.

Figure 1.19 Structure of Eslaminejad modified alginate for use as a cell scaffold. Alginate gels have also been used while studying the dedifferentiation process observed in chondrocytes. The phenotype expressed by chondrocytes is heavily reliant on the environment surrounding the cell. When chondrocytes are transferred from the *in vivo* environment into *in vitro* two dimensional culture the cells undergo dedifferentiation. This process changes the morphology of the cell from rounded to adherent and the genetic phenotype from chondrocyte to fibroblastic. When the
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Chondrocytes are transferred back into a three dimensional environment, either in vitro or in vivo, they reacquire the chondrocyte specific phenotype and produce cartilage extracellular matrix. Studies have shown that inhibition of ERK can slow the dedifferentiation process in vitro. The dedifferentiation of chondrocytes can be slowed by culture in alginate gels. Studies showed that it was possible to culture primary chondrocytes for up to 28 days in alginate beads before dedifferentiation occurred. Primary chondrocytes in alginate proliferated at a lower rate than in two dimensional culture but produced a higher level of cartilaginous matrix such as GAG and collagen type II. This supported the theory that alginate helped to prevent dedifferentiation of chondrocytes.

As all the above studies have shown alginate is an exceptionally useful tool for tissue engineering. The disadvantage of low cell attachment can be easily overcome. Its mild and reversible gelation and easy handling and storage properties make it an ideal bioscaffold particularly for mimicking, replacing and repairing cartilage sites.

1.8 Embryonic Stem Cells

Embryonic stem cells are pluripotent continuous cell lines which are isolated from fetal tissue of a preimplantation embryo. Embryonic stem cells are isolated at the blastocyst stage of embryogenesis. At this point the cells are pluripotent allowing them to differentiate into most cell types. After the blastocyst is formed, gastrulation occurs in which the stem cells form the three germ layers mesoderm, ectoderm and endoderm. Cells isolated at this stage can only differentiate to form the cells derived from the germ layer they are in. Cells isolated from the ectoderm differentiate to form the nervous system and the epidermis. Cells found in the mesoderm form muscle and blood cells, and cells isolated from the endoderm form the lung, thyroid and pancreas.

Embryonic stem cells were first discovered in 1970 when Solter grafted embryos into adult mice and observed that multidifferentiated tumours were produced. Further studies showed that any undifferentiated cells could be isolated and cultured in vitro while maintaining pluripotency. In order to maintain pluripotency the cells needed to be grown on a layer of ‘feeder’ cells which were mitotically inactivated.
Maintaining the pluripotency of the embryonic stem cells does not happen automatically and is highly dependent on the presence of nutrients and growth factors present in the cell media. Smith and Hopper discovered that the presence of a feeder layer of cells in co-culture was not necessary to maintain pluripotency in embryonic stem cells derived from mice, if the cells were cultured in the presence of leukemia inhibitory factor (LIF). This discovery paved the way for creating interactive scaffolds for stem cell growth.

Stem cells are hard to maintain in culture as they are very responsive to the surfaces to which they adhere. The cells cannot be grown on two dimensional treated tissue culture plastic or glass like most other cell types. The most common two dimensional culture of embryonic stem cells in vitro is to grow the cells on gelatin coated tissue culture plastic. This allows the cells to maintain their pluripotency. Although this is helpful in vitro, gelatin cannot form a three dimensional scaffold under physiological conditions due to its thermoresponsive nature.

Any material used as a scaffold for stem cell growth needs to be carefully selected. The cells need to be able to adhere to and grow on the scaffold but the scaffold can also be used to maintain pluripotency or direct differentiation. Several studies have been carried out showing the stiffness of the support used for cell growth can affect the cells as strongly as the chemical groups incorporated into the scaffold. Ying et al recently reported that altering the stiffness of the gel used as a cell scaffold increased mesenchymal stem cell differentiation in a stiffness dependent manner. Cooper White et al also showed that the stiffness of the gels had strong effects on the mesenchymal stem cells. Studies showed that this was related to the development of actin formation on the gels (Figure 1.20). The images clearly show that the cells adhered best on stiffer substrates and that coating the gels with different proteins had little effect. The stiffness of the gels directly influenced differentiation of the cells with myogenic differentiation observed on all gels that had a stiffness of 9 kPa or more. Osteogenic differentiation was dependent on the stiffness of the gel and the protein coating, differentiation occurred at stiffness of 25 kPa or greater and in the presence of collagen type I and fibronectin coatings.
As we have seen previously with primary cell cultures alginate is a good cell scaffold and a useful biomaterials tool. As alginate is not isolated from mammalian tissue it has been hypothesised that it should not interact adversely with embryonic stem cells. Mantalaris et al cultured undifferentiated E14Tg2a embryonic stem cells isolated from murine embryos in low viscosity alginate doped with porcine gelatin. The cells were mixed with the gel solution and formed into small beads by dropping into calcium chloride (100 mM) solution. The beads were then incubated in a bioreactor. The cells proliferated inside the bead, and live dead staining showed the cells were live and viable. Mineralisation of the samples began to occur significantly at day 15 and increased to day 30. The alkaline phosphatase activity also decreased during this period. Immunocytochemistry showed that pluripotency was maintained at day 3, but that differentiation had occurred at day 8, 3 days after the removal of LIF. Further studies at days 22 and 29 showed that extensive mineralisation had occurred.

Yarmush et al used alginate modified with poly-L-lysine (PLL) as a scaffold to grow D3 cells derived from mice. Alginate (2.2 % wt/vol) was formed with calcium free DMEM media and used to encapsulate cells. The gel solution was dropped into calcium chloride (100 mM) bath to crosslink the gel, forming alginate beads containing the cells. The beads were then incubated in poly-L-lysine. Live/ dead studies showed that the encapsulation did not harm the cells. Further studies showed that the cell population increased over the 14 day period, and that > 95 % of the cells remained viable. Encapsulation of the cells in alginate did not induce differentiation of the cells. After differentiation of the stem cells was induced, urea secretion and
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intracellular albumin levels both increased. This showed that the alginate beads can also support differentiated stem cells.

Alginate has been shown to be a successful tissue scaffold for stem cells, maintaining the pluripotency of the cells and supporting cell growth through the differentiation process and of differentiated cells. This opens up the alginate stem cell constructs to investigation for tissue engineering and biomedical applications.

1.9 Project Aims and Rationale

The aim of this work is to create a fully defined system formed from liposomes and nanoparticles which can act as an interactive scaffold for drug delivery to cells in vitro. The building blocks of this system are created from; 1) thermally sensitive liposomes which will be stable under physiological conditions but will release encapsulated material on the receipt of an external trigger. On the receipt of the trigger the membrane of the liposome is permeabilised allowing diffusion of the contents across the membrane, while the liposomal structure remains intact; 2) Magnetic nanoparticles, which will disperse in an aqueous environment and can interact with the external surface of the liposomes and serve as a stimuli sensitive trigger; 3) An aggregation process and methodology which will allow aggregation of liposome-nanoparticle assemblies, to form selectively and without interference from the surrounding biological environment and 4) a three dimensional scaffold which will support both liposomes and cell growth.

Figure 1.21 Schematic showing vesicle gel formation
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Based on this rationale the main research aims were defined.

- To synthesise a linking compound, which would allow magnetic nanoparticles to interact with biotin.
- To obtain biotin mediated aggregation of liposomes and magnetic nanoparticles, which can undergo magnetically triggered release.
- To find a hydrogel which can support the liposome-nanoparticle aggregates and act as a cell scaffold.
- To investigate the interactions of the liposomes and the nanoparticles with cells.
- To encapsulate and release biological cues magnetically, to trigger a response from the cells grown in the scaffold.
- To study the interactions between vesicle gels and embryonic stem cells, and to try to achieve magnetically triggered differentiation.
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Chapter 2

Materials and Methods

In this chapter all experimental procedures not already detailed in full in the following chapters are reported.
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2.1 Materials
DMPC, DPPC, biotin tagged DPPE and Rhodamine tagged DHPE were obtained from Avanti Lipids USA.
Gelatin, Chitosan, Sodium alginate, PEI, 5/6-carboxyfluorescein, calcium chloride, 3-(N-morpholino)propanesulfonic acid, DMSO, Trypan Blue, resazurin sodium salt ‘alaran blue’, bovine serum albumin (BSA), fluorescein isothiocyanate conjugated phalloidin (FITC-phalloidin), glutaraldehyde, dimethylthiazolylidiphenyltetrazolium bromide (MTT), paraformaldehyde (PFA), diamidinophenylindole (DAPI), Triton X-100, fluorescein isothiocyanate conjugated dextran (FITC-Dextran) nickel chloride, sodium hydroxide, biotin, N,N-Diisopropylethylamine (DIPEA), dimethylformamide (DMF), Calcein Blue and EDTA were all purchased from Sigma Aldrich, UK.
L-Glutamine, penicillin/streptomycin, fetal bovine serum (FBS), trypsin-EDTA preparation, phosphate buffered saline (PBS) and Dulbecco’s modified Eagle’s medium (DMEM) F12 media were all obtained from PAA, Yeovil, UK.
Tissue culture flasks, multiwall plates, ProLong Gold antifade reagent and Live/Dead assay, were all purchased from Invitrogen, Paisley, UK.
3T3 cells and C2C12 myoblast cells were obtained from the ECACC, Bovine Chondrocyte cells isolated from the metacarpal phalangeal joint were a kind gift from Dr Rachel Lalley, E14 cells were donated to the project by the Merry Group, University of Manchester. E Cad(-/-) cells were a kind gift from Dr Chris Ward, University of Manchester.

2.2 Basic Methods
NMR spectra were recorded using Bruker DPX 300, 400 and 500 instruments in deuterated solvents (CDCl₃ , CD₂OD), calibrated to the NMR solvent trace, 189 and assigned with the aid of ¹H, ¹³C, COSY and DEPT 135. Coupling constants are in Hertz (Hz) and multiplicities indicated with the appropriate abbreviations: singlet (s), doublet (d), triplet (t), double doublet (dd) double double doublet (ddd) and multiplet (m). Mass spectra (ES and HRMS) were taken using Micromass Prospec and Micromass Platform spectrometers. Liposomes were formed by extrusion through Liposofast polycarbonate membranes. Fluorescence spectroscopy was carried out on a
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Perkin Elmer LS55 luminescence spectrometer and a BMG labtech Fluorostar Optima plate reader. UV-vis spectroscopy was monitored using a JASCO v-660 spectrophotometer at 400 nm / min and a Multiskan Ascent plate reader. Transmision electron microscopy was performed on a Joel 1220 transmission electron microscope coupled to a high resolution GATAN ORIUS CCD camera. Induction heating was carried out with a water cooled EASYHEAT 0224 induction heater (2.0 kW, 150-400 kHz) with an EASYHEAT 300P work head. Neodymium iron boron (NdFeB) magnets either (5350 G, 23 mm x 20 mm x 6 mm) or N42 (4700 G, 3 mm x 13 mm) were purchased from emagnets UK, Sheffield.

2.3 Lipsome Formation

2.3.1 DMPC:DPPC vesicles encapsulating 5/6-carboxyfluorescein
Liposomes were formed from DMPC (1.27 mg, 1.73x10^{-6} mol), DPPC (12.55 mg, 1.71x10^{-5} mol) and biotin tagged DHPE (0.2 % mol/mol). The lipids were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. The lipid film once formed, was hydrated in 0.05 M 5/6-carboxyfluorescein in 3-(N-morpholino)propanesulfonic acid buffer at pH 7.4 (1 mL), heated to 40 °C, vortex mixed and extruded 19 times through a 800 nm polycarbonate membrane at 40 °C, which gave vesicles of ~800 nm diameter encapsulating 5/6-carboxyfluorescein.

2.3.2 Formation of DPPC vesicles encapsulating 5/6-carboxyfluorescein
Liposomes were formed from DPPC (14.55 mg, 1.9x10^{-5} mol) and biotin tagged DHPE (0.2 % mol/mol). The lipids were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. Once the lipid film was formed was hydrated in 5/6-carboxyfluorescein in MOPs buffer at pH 7.4 (0.05 M, 1 mL), heated to 50 °C, vortex mixed and extruded 19 times through a 800 nm polycarbonate membrane at 50 °C, which gave vesicles of ~800 nm diameter encapsulating 5/6-carboxyfluorescein.

2.3.3 Formation of rhodamine tagged vesicles
DPPC (14.55 mg, 1.9x10^{-5} mol) and biotin-DHPE (0.2 % mol/mol) were dissolved in a cholorform solution of rhodamine tagged DHPE (0.1 % mol/mol). The solvent was
removal in vacuo and the lipid film dried under vacuum for 60 minutes. The film was then resuspended in PBS (1 mL) and the flask heated to 50°C and vortex mixed until the film was entirely resuspended. The lipid solution was extruded 19 times through an 800 nm polycarbonate membrane at 50 °C, which formed vesicles of ~800 nm diameter.

2.3.4 Formation of DPPC liposomes encapsulating FITC-dextran.
DPPC (14.55 mg, 1.9x10⁻⁵ mol) and biotin-DHPE (0.2 % mol/mol) were dissolved in chloroform. The solution was removed under reduced pressure and the lipids were resuspended in a solution of FITC dextran (4 kDa, 1 mg/mL) in PBS (1 mL). The flask was heated to 50 °C and vortex mixed. The mixture was then extruded 19 times through an 800 nm polycarbonate membrane.

2.3.5 Formation of DPPC liposomes encapsulating ascorbic acid-2-phosphate.
DPPC (14.55 mg, 1.9x10⁻⁵ mol) and biotin-DHPE (0.2 % mol/mol) were dissolved in chloroform. The solution was removed under reduced pressure and the lipids were resuspended in a solution of ascorbic acid-2-phosphate (3.4 mM) in PBS (1 mL). The flask was heated to 50 °C and vortex mixed. The mixture was then extruded 19 times through an 800 nm polycarbonate membrane.

2.4 Magnetic Nanoparticles
2.4.1 Magnetic nanoparticle synthesis
Is detailed in Chapter 4 (S.3)

2.4.2 Synthesis of N-biotinyl-(3,4-dibenzyloxyphenylethylamide(protected))
Is explained in Chapter 4 (S.2)

2.4.4 Coating magnetic nanoparticles
The magnetic nanoparticles (25 mg) were suspended in deoxygenated methanol and N-biotinyl-(3,4-dihydroxyphenylethylamide) (6 mg, 1.58x10⁻⁵ mol) was added. The mixture was sonicated for 3 hours under nitrogen. The coated nanoparticles were
magnetically sedimented, using a NdBFe magnet. The sedimented nanoparticles were washed and resuspended in deoxygenated methanol, the repeated washing and magnetic sedimentation removed any non-chelated biotin-dopamine from the system. The solvent was removed in vacuo and the coated nanoparticles were stored under nitrogen.

2.4.5 Imaging magnetic nanoparticles using transmission electron microscopy
TEM samples were prepared by dispersing uncoated nanoparticles (1.2 mg/mL) in water (1 mL) by sonication for 2 hours and then the solution was placed on a carbon coated copper TEM grid. TEM imaging was carried out on a Joel 1220 TEM microscope.
TEM samples of coated nanoparticles were prepared using the same method, but using coated nanoparticles.

2.4.6 Dynamic light scattering of magnetic nanoparticles
Dynamic light scattering was carried out on a Wyatt Technologies DynaPro DLS instrument. Coated magnetic nanoparticles (1.2 mg) were added to distilled water (1 mL) and the mixture was sonicated for 2 hours at 25 °C which created a suspension which was diluted using distilled water to give a 6.2x10^{-7} mg/mL suspension (5 mL). Analysis gave an average 12.5 ± 1.5 nm diameter particle size.

2.5 Vesicle-nanoparticle assemblies
2.5.1 Aggregation and purification of vesicles and magnetic nanoparticles
The vesicle suspensions were added to coated magnetic nanoparticles (200 μL of a 1.2 mg/mL suspension in PBS) and avidin (20 μL of 10 mg/mL in PBS). The mixture was left to aggregate for 60 minutes. The vesicle-nanoparticle suspensions were then magnetically sedimented using an NdFeB magnet (5350 G) until a compact plug of magnetic material had formed at the bottom of the vial and the supernatant solution was visually free of turbidity. As much of the supernatant was removed as possible, without disturbing the vesicle-nanoparticle plug (typically 80
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% of the volume), and replaced with an equal volume of the appropriate buffer solution. Brief vortex mixing regenerated the vesicle suspension. This procedure was repeated at least 6 times and until the concentration of unencapsulated material was < 0.5% of the initial concentration.

2.5.2 Purification of liposomes
Liposomes without magnetic nanoparticles present were purified using PD-10 sephadex column. The column was equilibrated using PBS (25 mL), and the liposome suspension diluted by PBS (2.5 mL) and added to the column. The liposomes were then eluted using PBS (3.5 mL) which separated any unencapsulated material.

2.5.3 Time dependent aggregation of vesicle-nanoparticle assemblies
Experimental details in Chapter 7 (Section 2)

2.6 Hydrogels
2.6.1 Formation of gelatin gel
Gelatin was dissolved in sterile PBS (1 % wt/vol) by stirring at 40 °C. The mixture was autoclaved to ensure sterility. The mixture was aliquoted and cooled to room temperature to allow gelation. After gelation had occurred the gels were washed with PBS.

2.6.2 Formation of chitosan gel
Chitosan was dissolved in aqueous sulfuric acid in sterile filtered water. H₂SO₄, pH 3 (0.5 mL), chitosan (2 % wt/vol) mixture was aliquoted and the gel was formed on the addition of NaOH (2 M, 1 mL) for 2 minutes, any remaining solution was removed and the gels washed with PBS.

2.6.3 Formation of chitosan:PEI gels
Chitosan:PEI gels were formed by mixing chitosan and PEI solutions which triggered gelation. Gel solutions were chitosan 2 % wt/vol in H₂SO₄ (0.25 mL, pH 3)
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and PEI (30 % wt/vol in sterile filtered water at pH 11, 0.25 mL). Chitosan (0.25 mL) and PEI (0.25 mL) were aliquoted together to trigger gelation. Any excess solution was removed and the gels were washed with PBS.

2.6.4 Formation of alginate gels
Sodium alginate (2 % wt/vol) was dissolved in sterile PBS and stirred at room temperature. The solution was autoclaved to remove impurities and aliquoted (0.5 mL). Calcium chloride solution was formed by dissolving calcium chloride in sterile PBS (5.5 mg, 100 mM) and filter sterilising. Calcium chloride solution was then added to the gel solution (0.5 mL) and incubated for 30 minutes. The excess calcium chloride was removed from the gel and the gel washed with sterile PBS.

2.6.5 Formation of alginate fibronectin gels
Sodium alginate (2 % wt/vol) was dissolved in sterile PBS and stirred at room temperature. The solution was autoclaved to remove impurities. Bovine fibronectin was added to the alginate solution (2.27x10^{-9} M) and the mixture aliquoted (0.5 mL). Calcium chloride solution was formed by dissolving calcium chloride in sterile PBS (5.5 mg, 100 mM) and filter sterilising. Calcium chloride solution was then added to the gel solution (0.5 mL) and incubated for 30 minutes. The excess calcium chloride was removed from the gel and the gel washed with sterile PBS.

All gels were sterilised under UV light for 30 minutes prior to cell culture.

2.7 3T3 Cell Culture
2.7.1 Cell media
All 3T3 fibroblast cell experiments were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) with the addition of Fetal Bovine Serum (10 %, 50 mL) and Penicillin/streptomycin antibiotics (1 %, 5 mL). The media was stored at 4 °C and warmed to 37 °C before use.
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2.7.2 Cell culture
Frozen cell suspension stored over liquid nitrogen in fibroblast media with 10 % DMSO (1 mL) were removed from the liquid nitrogen, vented and warmed to 37 °C. The cell suspension was then added to cell media (4 mL) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed leaving a cell pellet which was resuspended in media (10 mL) and added to a T75 culture flask. The media on the flask was changed every 48 hours and the flask was split when cell confluency reached 70-80 %.

The media was removed from the flask and the cell monolayer washed twice with PBS. Trypsin.EDTA (0.105 mM, 3 mL) was added to the flask which was incubated at 37 °C for 5 minutes. Media (2 mL) was then added to the flask and pipetted to ensure removal of the cells from the flask, and the cells centrifuged at 1500 rpm for 5 minutes. The cells were then resuspended in fresh media (10 mL) and split 1:3 ratio and added to new flasks with fresh media (7 mL).

Cryostore of cells was obtained by removing the media from a confluent flask and washing twice with PBS. Trypsin.EDTA (0.105 mM, 3 mL) was added to the flask which was incubated for 5 minutes. Media (2 mL) was then added to the flask and the cells centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the cell pellet was resuspended in media (10 mL) with DMSO (10 %, 1 mL), aliquoted into cryovials (1 mL) and placed in a Mr Frosty at -80 °C for 48 hours and then transferred to liquid nitrogen.

2.7.3 3T3 cell culture in gels
3T3 cells grown in gelatin gels were harvested from a confluent flask of cells. A solution of cells was centrifuged at 1500 rpm and the supernatant removed. The cell pellet was then resuspended in a gelatin solution (1 % wt/vol) in PBS. The gels were set at room temperature. The cells grown in chitosan gels were harvested from a confluent flask and centrifuged at 1500 rpm and the supernatant was removed. The cells were then resuspended in chitosan solution (2 % wt/vol, 0.5 mL). The mixture was gelled on the addition of PBS (0.5 mL). The cells grown in chitosan:PEI gels were formed by suspending cells in a PEI solution (30 % vol/vol, 0.25 mL). The
solution was then gelled on the addition of chitosan solution (0.25 mL, 2 % wt/vol). Cells cultured in alginate gels were suspended in a solution of sodium alginate (0.5 mL, 2 % wt/vol) which was then cured using calcium chloride (0.5 mL, 100 mM) the excess calcium chloride was removed and the gels washed with PBS. Cells grown in the alginate fibronectin matrix were prepared in the same way, but the alginate solution was doped with fibronectin from bovine plasma ($2.27 \times 10^{-9}$ mol). All gels were suspended in cell media (1 mL) and incubated at 37 °C and 5 % CO$_2$.

2.8 Chondrocyte Cell Culture

2.8.1 Cell media

All bovine chondrocyte cell experiments were cultured in DMEM:F-12 (1:1-Dulbecco’s Modified Eagle Media: Nutrient Mixture (Ham)) with the addition of Fetal Bovine Serum (10 %, 50 mL), Penicillin/streptomycin antibiotics (1 %, 5 mL), ascorbic acid-2-phosphate (2.5 g, 0.0172 M) unless otherwise stated. The media was stored at 4 °C and warmed to 37 °C before use.

2.8.2 Cell culture

Frozen cell suspension stored over liquid nitrogen in chondrocyte media with 10 % DMSO (1 mL) were removed from the liquid nitrogen, vented and warmed to 37 °C. The cell suspension was then added to cell media (4 mL) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed leaving a cell pellet which was resuspended in media (10 mL) and added to a T75 culture flask. The media on the flask was changed every 48 hours and the flask was split when cell confluency reached 70-80 %.

The media was removed from the flask and the cell monolayer washed twice with PBS. Trypsin.EDTA (0.105 mM, 3 mL) was added to the flask which was incubated at 37 °C for 5 minutes. Media (2 mL) was then added to the flask and pipetted to ensure removal of the cells from the flask surface, and the cells centrifuged at 1500 rpm for 5 minutes. The cells were then resuspended in fresh media (10 mL) and split.
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1:3 ratio and added to new flasks with fresh media (7 mL). Chondrocytes were used up to passage 6.
Cryostore of cells were created as described in section 2.7.2

2.8.3 Culture of chondrocytes in gel scaffolds
Bovine chondrocyte cells were grown in gelatin gels were harvested from a confluent flask of cells. A solution of cells was centrifuged at 1500 rpm and the supernatant removed. The cell pellet was then resuspended in a gelatin solution (1 % wt/vol) in PBS. The gels were set at room temperature. The cells grown in chitosan gels were harvested from a confluent flask and centrifuged at 1500 rpm and the supernatant was removed. The cells were then resuspended in chitosan solution (2 % wt/vol, 0.5 mL). The mixture was gelled on the addition of PBS. The cells grown in chitosan:PEI gels were formed by suspending cells in a PEI solution (30 % vol/vol, 0.25 mL). The solution was then gelled on the addition of chitosan solution (2 % wt/vol, 0.25 mL). Cells cultured in alginate gels were suspended in a solution of sodium alginate (0.5 mL, 2 % wt/vol) which was then cured using calcium chloride (0.5 mL, 100 mM) the excess calcium chloride was removed and the gels washed with PBS. Cells grown in the alginate fibronectin matrix were prepared in the same way, but the alginate solution was doped with fibronectin from bovine plasma (2.27x10^9 mol). All gels were suspended in cell media (1 mL) and incubated at 37 °C and 5 % CO₂.

2.9 Myoblast cell culture
2.9.1 Cell media
All myoblast cell experiments were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) with the addition of Fetal Bovine Serum (10 %, 50 mL) and Penicillin/streptomycin antibiotics (1 %, 5 mL) unless otherwise specified. The media was stored at 4 °C and warmed to 37 °C before use.
2.9.2 Cell culture
Frozen cell suspension stored over liquid nitrogen in DMEM with 10 % DMSO (1 mL) were removed from the liquid nitrogen, vented and warmed to 37 °C. The cell suspension was then added to cell media (4 mL) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed leaving a cell pellet which was resuspended in media (10 mL) and added to a T75 culture flask. The media on the flask was changed every 24 hours and the flask was split when cell confluency reached 70-80%.

The media was removed from the flask and the cell monolayer washed twice with PBS. Trypsin.EDTA (0.105 mM, 3 mL) was added to the flask which was incubated at 37 °C for 5 minutes. Media (2 mL) was then added to the flask and pipetted to ensure removal of the cell monolayer from the flask, and the cells centrifuged at 1500 rpm for 5 minutes. The cells were then resuspended in fresh media (10 mL) and split 1:3 ratio and added to new flasks with fresh media (7 mL).

A cryostore of cells was created as described in Section 2.7.2

2.10 Cell Assays

2.10.1 Trypan blue exclusion assay
After a cell suspension has been obtained 10 μL was taken and added to a sterile eppendorph tube. Trypan Blue stain 0.85 % in saline (10 μL) was added to the eppendorph tube and mixed by pipette. The mixture was then added to a haemocytometer and the cells counted as dead (having taken up trypan blue) or live (having not).

2.10.2 Alamar blue metabolic activity assay
Alamar blue was performed in 24 well tissue culture plates. The media was removed and fresh media (1 mL) and alamar blue solution (100 μL) were added to each well in the plate. The plate was then incubated at 37 °C for 2 hours. The media was removed from the plate and added to a 96 well clear bottom plate in three aliquots (200 μL) for each well and the fluorescence read at 590 nm.
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2.10.3 DNA assay
DNA assay was performed in 24 well tissue culture plates, cell samples had been previously used for the alamar blue assay unless otherwise stated. The media was removed and sterile water (1 mL) was added. The plates were placed at -80 °C for 72 hours and then incubated at 37 °C. The plates were refrozen at -80 °C and incubated again at 37 °C this was repeated twice. The solution in the plates was then added to a 96 well clear bottom plate in 3 aliquots (50 μL) from each well. TNE buffer (50 μL) was added to each well of the 96 well plate and Hoescht 33342 stain (100 μL) was also added. Fluorescence was then read at 460 nm.

2.10.4 MTT assay
The MTT assay was carried out in a 24 well tissue culture plate. All media was removed from the plate and the cells were suspended in fresh media (1 mL). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS (1 mg/mL, 100 μL) was added to each well and the plates incubated at 37 °C for 3 hours. The culture media was removed and the cells suspended in isopropyl alcohol (600 μL) and shaken at 25 °C for 30 minutes. The solution was then added to a 96 well clear bottom plate in three aliquots (200 μL) from each well and the absorbance read at 540 nm.

2.11 Liposome-nanoparticle assemblies in biological systems
2.11.1 Liposome stability under Culture Conditions
Suspensions of DPPC liposomes with 5/6-carboxylfluorescein encapsulated were purified using sephadex method (Section 2.5.2) and aliquoted into tissue culture plastic well plate (25 μL) diluted in PBS (1 mL). The solutions were then incubated under cell culture conditions at 37 °C and CO₂ (5 %, vol/vol). Aliquots of the supernatant were removed (200 μL) and the fluorescence measured using a Fluorostar optima fluorescence plate reader every 60 minutes for 5 hours. After this time Triton X-100 (1 % vol/vol) was added in PBS (1 mL) to each well and the plate incubated at room temperature for 20 minutes. Aliquots of the solution (200 μL) were taken and the fluorescence measured at 520 nm after excitation at 480 nm.
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2.11.2 Cell-liposome interactions
Experimental work detailed in Chapter 3 (Section 2)

2.11.3 Imaging cell-vesicle interactions
Experimental work detailed in Chapter 3 (Section 2)

2.11.4 Flow cytometric analysis of 3T3 cells with liposomes
Experimental work detailed in Chapter 3 (Section 2)

2.11.5 Vesicle gels imaging using scanning electron microscopy
Vesicles encapsulating 5/6-carboxyfluorescein were aggregated with magnetic nanoparticles and avidin as previously described (Section 2.5.1). The sedimented vesicle-magnetic nanoparticle assemblies were resuspended in alginate (1 mL, 2 % wt/vol) and added to a glass vial in layers ~2 mm thick. The solutions were cured on the addition of calcium chloride (1 mL, 0.1 M). Vesicle gels were dehydrated using an increasing concentration of ethanol from 50 to 100 % ethanol in 0.1 M phosphate buffer and left to dry overnight. The samples were mounted on aluminium stubs via an adhesive carbon sticker and sputter coated on an Edwards Sputter Coater E150b under an argon gas atmosphere. Scanning electron microscopy was carried out on Zeiss EVO SEM.

2.11.6 Cell-vesicle gels imaging using environmental scanning electron microscopy
DPPC (14.55 mg, 1.9x10^-5 mol), biotin-DHPE (0.2 % mol/mol) and rhodamine tagged DHPE (0.1 % mol/mol) were dissolved in chloroform (1 mL). The solvent was removed in vacuo and the lipid film dried under vacuum for 60 minutes. The film was then resuspended in PBS (1 mL), the flask heated to 50 °C and vortexed until the film was entirely resuspended. The vesicle suspension was added to coated magnetic nanoparticles (200 μL of a 1.2 mg/mL) and avidin (50 μL of 10 mg/mL). The mixture was left to aggregate for 60 minutes. The vesicle aggregates were then magnetically sedimented using NdBFm magnet and the supernatant removed. The
magnet was removed and the aggregates resuspended in PBS (1 mL), resedimented and washed 3 more times. After the last sedimentation the vesicle-nanoparticle aggregates were resuspended in alginate (1 mL, 2 % wt/vol). The vesicle gel (0.5 mL) was added to four wells of a 24 well plate. Calcium chloride (0.5 mL, 0.1 M) was added to the well and the gel left to cure for 30 minutes. The excess calcium chloride and the gel block washed with PBS. 3T3 cell monolayer at 70 % confluency was separated using Trypsin.EDTA (0.105 mM, 3 mL) for 5 minutes at 37 °C and fresh media (2 mL) was added. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant removed. The cell pellet was resuspended in fresh media (5 mL) and a 20 μL sample aliquoted into a haemocytometer to ascertain a cell count. 3T3 cells (20,000) were then seeded on the top of the vesicle gel block and the gel block resuspended in fresh media (1 mL) was added. The samples were incubated at 37 °C for 14 hours. The media was removed and the samples were washed with PBS (2 mL). Samples were transferred to the scanning electron microscope and imaged without stains or coatings. Imaging was carried out on a Zeiss Evo 60 Extended Pressure scanning electron microscope.

2.11.7 Patterning alginate vesicle gels
Experimental work detailed in Chapter 5 (Section 2)

2.11.8 Rheometrical studies of vesicle gels
Experimental work detailed in Chapter 5 (Section 2)

2.11.9 Interactions between cells and magnetic nanoparticles
3T3 cells were harvested and centrifuged at 1500 rpm for 5 minutes. The media was removed and the cells were resuspended in fresh media. The cells were seeded into a 6 well plate at a density of 25,000 cells per well and coated magnetic nanoparticles were added to the well (200 μL, 1.2 mg/mL). The samples were incubated at 37 °C and CO₂ (5 % vol/vol) for 3 days. The media was then removed and the cells were harvested and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the cell-nanoparticle pellet was resuspended in fresh media. The cells were
replated onto 6 well plates and cultured over an NdBFe magnet overnight. The cells were then counted using trypan blue exclusion assay.

2.12 Ascorbic acid-2-phosphate Vesicle Gels

2.12.1 Release from vesicle gels.
Vesicles encapsulating ascorbic acid-2-phosphate were used to create vesicle gels. Vesicle-nanoparticle gels were formed from alginate as previously described (Section 2.3.4). Gel blocks were formed at a 5x5x1 mm size by pouring the vesicle-gel solution into 25x5x1 mm trough and cured using calcium chloride (0.1 M) before cutting the blocks. The gel block was washed with PBS (2 mL) and placed in a UV-vis cuvette with PBS (2 mL). The cuvette was then exposed to an AMF (392 kHz) for 240 s, then the cuvette transferred to a JASCO v-660 spectrophotometer. The spectra was measured at 260 nm and the concentration was determined by interpolation into a standard curve. Thermal release was obtained by heating the cuvette to 50 °C for 4 minutes before the spectra was measured.

Vesicles encapsulating ascorbic acid-2-phosphate were used to create vesicle gels. Gel blocks were formed as previously described. Analysis was carried out using a modification of the published method of Kazakeviciene et al. Ascorbic acid-2-phosphate containing vesicle-gel blocks (25 mm³) were added to glass vials and suspended in PBS (2 mL). The samples were subjected to a 4 minute alternating magnetic field (AMF) pulse at 392 kHz and then incubated at room temperature. At each timepoint, aliquots (200 μL) were removed, from which smaller aliquots (3 × 50 μL) were taken and transferred to a 96 well plate (in 3 separate wells). Then alkaline phosphatase (from bovine intestinal mucosa, lyophilized powder, 2,000 – 4,000 DEA units/mg protein, 979 units/mg solid) in PBS (0.09 nM, 50 μL) was added to each well and the plate incubated at 37 °C for 30 minutes. Starchtriiodide solution (~20 μM I3⁻ in PBS, 50 μL) was added to each well, the absorbance measured at 562 nm. A standard curve was created by measuring the decrease in the same I3⁻/starch absorbance after the addition of known concentrations of ascorbic acid-2-phosphate that had been incubated with alkaline phosphatase. The absorbance from each of the gel samples was interpolated into the standard curve to give the concentration of released ascorbic acid-2-phosphate. Thermal release was obtained
2.12.2 Ascorbic acid-2-phosphate containing vesicle gels as cell scaffolds

Aliquots of sodium alginate solution that contained vesicle gels with encapsulated ascorbic acid-2-phosphate (0.5 mL) were transferred into glass vials and sterilised under UV light for 1 hour. A chondrocyte cell monolayer at 70% confluency was separated using trypsin/EDTA solution (0.105 mM, 3 mL) for 5 minutes at 37 °C and fresh media (DMEM:F12 (1:1 – Dulbecco’s Modified Eagle media: Nutrient mixture (Ham) with 10% fetal bovine serum and 1% penicillin streptomycin, 2 mL) added. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant removed. The cell pellet was resuspended in fresh media (5 mL) and an aliquot (20 μL) transferred into a haemocytometer to ascertain a cell count. Aliquots of sodium alginate solution containing vesicle-nanoparticle assemblies (0.5 mL) were then seeded with 25,000 cells added to each aliquot (25,000 cells in 0.5 mL, 50 cells/mm$^3$). The samples prepared had chondrocytes mixed with:

- Liposome-nanoparticle assemblies encapsulating ascorbic acid-2-phosphate in sodium alginate. After gelation with calcium chloride (as above), the gel block (500 mm$^3$) was suspended in media (1 mL) without ascorbic acid-2-phosphate present. Samples were either exposed to an AMF or incubated in the absence of an AMF.

- Liposomes encapsulating ascorbic acid-2-phosphate in sodium alginate. After gelation with calcium chloride (as above), the gel block (500 mm$^3$) was suspended in media (1 mL) without ascorbic acid-2-phosphate present. Samples were exposed to an AMF pulse (3 min).

- Liposomes encapsulating ascorbic acid-2-phosphate and uncoated magnetic nanoparticles in sodium alginate. To remove unencapsulated material from these non-magnetic vesicles, the vesicle suspension was purified by sephadex method. The suspension obtained after passing through the sephadex column was concentrated using centrifugation (1200 rpm for 10 minutes; test runs on 5/6-CF containing DPPC vesicles showed this treatment did not cause vesicle rupture), enough supernatant was removed to give a solution that was 20 mM lipid after resuspension in sodium alginate solution. After gelation with calcium chloride (as above), the gel block (500...
mm$^3$) was suspended in media (1 mL) without ascorbic acid-2-phosphate present. Samples were exposed to an AMF pulse (3 minutes).

- Calcium alginate only. After gelation with calcium chloride (as above), the resulting gel block was suspended in media (1 mL) with and without ascorbic acid-2-phosphate present (173 mM). Samples were exposed to an AMF pulse (3 min). The samples were incubated at 37 °C with 5 % CO$_2$. At 3, 7, 10 and 14 days, samples to be analyzed for the respective time points were removed from the incubator and placed at -80 °C for at least 48 hours. The samples were then warmed to 25 °C and the media removed. Pepsin (0.1 mg/mL, 1 mL) in acetic acid (0.5 M, pH 3) was added to each sample followed by incubation at 4°C for 12 hours. Aliquots of the samples (50 μL) were then transferred into Eppendorf tubes and sterile water (50 μL) was added to each. Sircol dye reagent (1 mL) was added to each sample and the tubes shaken for 30 minutes at 25 °C. A standard curve for collagen was obtained by adding 5 μL, 10 μL, 25 μL and 50 μL of Sircol collagen standard (1 mg/mL collagen) to Eppendorf tubes and were made up to 100 μL with sterile water. Sircol dye reagent (1 mL) was added to each standard and shaken for 30 minutes at 25 °C. The samples and standards were then microcentrifuged at 10,000 $\times$ g for 10 minutes. The solutions were then removed from each Eppendorf tube and the pellets in the Eppendorf tube dried. Sircol alkali reagent (1 mL) was added to each tube and vortex mixed for 10 minutes to dissolve the pellets in the reagent. The samples were then added to a 96 well clear bottom well plate in three aliquots (200 μL each) from each sample and the absorbance measured at 540 nm. The concentration was determined by interpolation into a standard curve.

2.12.3 Imaging chondrocyte-vesicle gels

Vesicle gels were formed as previously described (Section 2.12.2). At day 7 and 14 the media was removed and the gels were washed using PBS. The samples were fixed using paraformaldehyde (4 % vol/vol, 2 mL) for 10 minutes. The PFA was removed and the sample washed using PBS. The cells were blocked using ICC block (goat serum (1 % vol/vol), bovine serum albumin (1 mg/mL, 0.1 % vol/vol) and Triton X-100 (0.1 % vol/vol)) for 45 minutes at room temperature. The samples were incubated with primary antibody mouse monoclonal anti-collagen I (ab90395)
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diluted (1:200) in ICC block solution (1 mL) for 60 minutes. The samples were then washed 10 times with PBS. The gel was then incubated in secondary antibody, goat anti mouse Alexa Fluor 488 diluted (1:1000) in ICC block solution (1 mL). The samples were then washed using PBS and incubated in DAPI (10 µL, 3 µL/mL) for 5 minutes and imaged using a Leica confocal microscope (LCS SC5). Samples imaged for collagen II were processed in exactly the same way but used primary antibody mouse monoclonal anti collagen II (ab3092) diluted (1:200) in ICC block solution (1 mL) and secondary antibody, goat anti mouse Alexa Fluor 546 diluted (1:1000) in ICC block solution (1 mL). The gels were washed using PBS and incubated in DAPI (10 µL, 3 µL/mL) for 5 minutes. The gels were then imaged using a Leica confocal microscope (LCS SC5).

2.13 Nickel Chloride Vesicle Gels as Cell Scaffolds

2.13.1 Formation of vesicle-nanoparticle encapsulating nickel chloride
Experimental work detailed in Chapter 5 (Section 2)

2.13.2 Release of nickel chloride from vesicle gels
Experimental work detailed in Chapter 5 (Section 2)

2.13.3 Effect of gel thickness on nickel chloride release
Experimental work detailed in Chapter 5 (Section 2)

2.13.4 Nickel encapsulated vesicle gels as cell scaffolds
Experimental work detailed in Chapter 5 (Section 2)

2.13.5 Imaging magnetically induced apoptosis
Experimental work detailed in Chapter 5 (Section 2)

2.13.6 Imaging the effect of nickel on cells
Experimental work detailed in Chapter 5 (Section 2)

2.13.7 Dual release of nickel chloride and calcine
Experimental work detailed in Chapter 5 (Section 2)
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2.14 Interactive Vesicle Gels

2.14.1 Encapsulation of 2-aminoacidone labelled heparin sulphate
Experimental work detailed in Chapter 7 (Section 2)

2.14.2 Release of 2-aminoacidone labelled heparin sulphate
Experimental work detailed in Chapter 7 (Section 2)

2.14.3 Encapsulation of MTT
Experimental work detailed in Chapter 7 (Section 2)

2.14.4 Release of MTT
Experimental work detailed in Chapter 7 (Section 2)

2.14.5 Glutamine encapsulated vesicle gels as cell scaffolds
Liposomes were formed from DPPC (14.55 mg, 1.9x10^5 mol) and biotin tagged DHPE (0.2 % mol/mol). The lipids were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. The lipid film formed was hydrated in L-Glutamine (200 mM, 1 mL) in PBS and then extruded 19 times through an 800 nm polycarbonate membrane at 50 °C to give vesicles of ~800 nm diameter. The liposomes were aggregated with coated magnetic nanoparticles (200 µL, 1.2 mg/mL) and avidin (20 µL, 10 mg/mL) for 60 minutes. The vesicle-nanoparticle aggregates were magnetically purified and resuspended in sodium alginate solution.

Myoblast cells were harvested and centrifuged at 1500 rpm for 5 minutes. The supernatant removed and the cells were resuspended in fresh media. The cells were then added to sodium alginate solution or sodium alginate solution containing L-glutamine encapsulated nanoparticles at a density of 20,000 per gel. The cell-gel suspension was added to glass culture well (0.5 mL) and cured using calcium chloride (0.5 mL, 100 mM). The excess solution was removed and the gels were washed with PBS. The gels with no vesicles present were incubated in media with L-glutamine present and media without L-glutamine present. The gels with vesicles present were incubated in media without L-glutamine present and either did or did
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not undergo an alternating magnetic field pulse. The plates were incubated at 37 °C and CO₂ (5 % vol/vol) and the alamar blue and DNA assays were run every 24 hours for 5 days.

2.14.6 MTT encapsulated in vesicle gels as cell scaffolds
Experimental work detailed in Chapter 7 (Section 2)

2.15 Embryonic Stem Cell
2.15.1 E14 stem cell culture
Experimental work detailed in Chapter 6 (Section 2)

2.15.2 Two dimensional culture of E14 cells on gel scaffolds
Experimental work detailed in Chapter 6 (Section 2)

2.15.3 Three dimensional culture of E14 cells on gel scaffolds
Experimental work detailed in Chapter 6 (Section 2)

2.15.4 EDTA mediated gel disruption
Experimental work detailed in Chapter 6 (Section 2)

2.15.5 Calcium dependence of alginate gel formation
Experimental work detailed in Chapter 6 (Section 2)

2.15.6 Ascorbic acid-2-phosphate mediated differentiation
Experimental work detailed in Chapter 6 (Section 2)

2.15.7 Neural differentiation
Experimental work detailed in Chapter 6 (Section 2)

2.15.8 E-Cad (−/−) cell culture
Experimental work detailed in Chapter 6 (Section 2)
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2.15.9 E-Cad<sup>(−/−)</sup> cell culture in media without LIF
Experimental work detailed in Chapter 6 (Section 2)

2.15.10 Encapsulation of SB 431542
Experimental work detailed in Chapter 6 (Section 2)

2.15.11 Release of SB 431542
Experimental work detailed in Chapter 6 (Section 2)

2.15.12 Magnetically induced differentiation of E Cad<sup>(−/−)</sup> cells
Experimental work detailed in Chapter 6 (Section 2)
Chapter 3
Adhesive interactions between cells and biotinylated phospholipid vesicles in alginate: towards new responsive biomaterials.
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J. Mater. Sci.-Mater. M.
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This chapter shows published material on the interactions between liposomes and cells in a cell culture environment.
Adhesive interactions between cells and biotinylated phospholipid vesicles in alginate: towards new responsive biomaterials

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Abstract Creating tissue-mimetic biomaterials able to deliver bioactive compounds after receipt of a remote and non-invasive trigger has so far proved to be challenging. The possible applications of such “smart” biomaterials are vast, ranging from subcutaneous drug delivery to tissue engineering. Self-assembled phospholipid vesicles (liposomes) have the ability to deliver both hydrophilic and hydrophobic drugs, and controlling interactions between functionalized vesicles and cells within biomaterials is an important step for targeted drug delivery to cells. We report an investigation of the interactions between thermally-sensitive and biotin-coated dipalmitoyl phosphatidylcholine vesicles and 3T3 fibroblast cells. The stability of these vesicles under physiological conditions was assessed and their interaction with the cell membranes of fibroblasts in media and alginate/fibronectin mixtures was studied. Stable vesicle-cell aggregates were formed in fluid matrices, and could be a model system for improving the delivery of remotely released drugs within vesicle-containing biomaterials.

Keywords: vesicle; phospholipid; liposome; 3T3 fibroblast; alginate; biotin

1 Introduction

Phospholipid vesicles are spherical self-assembled capsules that have been widely studied due to their ability to mimic cell membranes and act as delivery vehicles for bioactive compounds like drugs [1]. Their membranes can be engineered in a variety of ways to
modulate their interaction with biological targets. Masking vesicle membranes with polyethylene glycol (PEG) lipids creates ‘stealth liposomes’ and inhibits their recognition by the immune system [2,3]. On the other hand, groups like antibodies or glycosaminoglycans can be appended to the exterior of vesicles to create drug carriers that will recognize specific cell types [4].

Phospholipid vesicles can act as active stores for both hydrophilic and hydrophobic molecules [5,6,7], which has generated extensive interest in their use as drug delivery vehicles in vivo and in vitro [1,8]. Targeting drug-containing vesicles to cells may improve efficacy of smart biomaterials by bringing the carrier and cell into proximity before release of the vesicle contents. However to be used in this manner, selective recognition of the vesicles by cells must be fully understood. A persistent problem in clinical trials is recognition by lymphocytes, leading to clearance of drug-containing vesicles from the body via the reticuloendothelial system (RES) before they reach their target cells [1,9,10]. After recognition, vesicles can be engineered to be endocytosed or to fuse with the cell membrane, for example cationic lipids can promote endocytosis while unsaturated phospholipids, e.g. with dioleoyl chains, can promote fusion with cell membranes [10,11,12]. Vesicles which are endocytosed are usually trafficked to endosomes and lysosomes where they are degraded and their contents destroyed, although some progress is being made developing vesicular carriers that are activated by the low pH in endosomes to release their contents [10].

In recent studies we have shown that assemblies of magnetic nanoparticles and thermally-sensitive vesicles can be used to store hydrophilic compounds, for example drug molecules, which can be specifically released by a magnetic or thermal trigger [6,14,15]. When the assemblies of magnetic nanoparticles and thermally-sensitive vesicles are incorporated into hydrogel materials, the released compounds can be used as biological actuators in vitro that initiate changes in surrounding cells cultured in the hydrogel. Ascertaining the presence and measuring the strength of interactions between vesicles and cells in this in vitro system is crucial. This may allow targeted cell kill, which has significance in aspects of tissue engineering, particularly for the removal of misdifferentiated stem cells from engineered tissue. It may also allow us to develop the potential of magnetic nanoparticle/thermally-sensitive conjugates as vesicle-based drug delivery systems in vivo; targeting and remote magnetic triggering of drug delivery to cells is of great interest in the future for clinical drug delivery. Thermally sensitive vesicles with an adhesive biotin coat
will form the next generation of our magnetic nanoparticle/vesicle assemblies [16]. Herein we report investigations into the interaction of these adhesive vesicles with 3T3 fibroblasts, a stable cell line that proliferates rapidly in fibronectin containing calcium alginate gels [16] and can be used as a model for anchorage dependent cells.

2 Materials and Methods

2.1 Biotinylated dipalmitoyl phosphatidylcholine vesicle (Bt-DPPC vesicle) preparation

Phospholipid vesicles (800 nm diameter) were formed from dipalmitoyl phosphatidylcholine (DPPC, 14.6 mg, 1.9 x 10^{-5} mol, Avanti Lipids) and 0.2 % mol/mol N-(biotinoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (Bt-DHPE, 200 µL of a 0.2 mM solution in chloroform, Avanti Lipids, USA). The lipids were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. The lipid film was rehydrated in phosphate buffered saline (PBS, 1 mL, Invitrogen). The solution was vortex mixed and heated to 50 °C to fully disperse the lipid film and form a suspension, which was then extruded (19 passes) at 50 °C through an 800 nm pore membrane using an Avestin Lipofast extrusion apparatus.

2.2 Preparation of rhodamine-tagged Bt-DPPC vesicles

Prepared as for standard Bt-DPPC vesicle preparation but the lipids were dissolved in rhodamine-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE) solution in chloroform (0.02 mM, 1 mL to give 0.1 % mol/mol), in the place of chloroform alone. The vesicles produced were sized by fluorescence microscopy and found to be (1.2 ± 0.6) µm in diameter.

2.3 Preparation of Bt-DPPC vesicles encapsulating either 5/6-carboxyfluorescein (5/6-CF) or fluorescein isothiocyanate-tagged dextran (FITC-dextran).

The lipid film was formed as described for standard vesicle preparation, however, the lipid film was rehydrated in a solution of either: 5/6-CF (0.05 M, Sigma UK) prepared in 3-({N-
morpholino)propanesulfonic acid buffer (MOPS, 1 mL) or FITC-dextran (4 kDa, 1 mg/mL) in PBS (1 mL). The lipid film was vortex mixed and extruded as described previously. These vesicles with encapsulated compounds were purified using a PD-10 Sephadex column which had been pre-equilibrated using PBS (25 mL). The vesicle suspensions (1 mL) were diluted to 2.5 mL with PBS, loaded onto the column and then eluted from the column with a further 3.5 mL of PBS.

2.4 Preparation of Bt-DPPC vesicles in alginate/fibronectin mixtures

Freshly prepared Bt-DPPC vesicles (1 mL) were centrifuged at 500 rpm for 3 minutes and the supernatant removed. The pellet was resuspended in sodium alginate solution (2 % wt/vol) and fibronectin solution (1 mg/mL) was added at 1 % vol/vol. The gel was cured by the infusion of CaCl₂ (0.1 M, 1 mL) through an 800 nm polycarbonate membrane into the sodium alginate/vesicle suspension.

2.5 Growth and maintenance of 3T3 fibroblast cultures

3T3 Fibroblast cells (ECACC, UK) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % vol/vol Fetal Bovine Serum (FBS) and antibiotic (1 % penicillin, 1 % streptomycin). Cells were passaged at 80 % confluency using trypsin/EDTA (25 %).

2.6 Assaying Bt-DPPC vesicle stability

Suspensions of Bt-DPPC vesicles with encapsulated 5/6-carboxyfluorescein (25 μL, total lipid concentration = 20 mM) were added to PBS (1 mL) in a 24 well culture plate (PAA) and incubated under cell culture conditions (37 °C and 5 % vol/vol CO₂). Aliquots of the supernatant were removed (200 μL) and the fluorescence was measured using a Fluorostar optima fluorescence plate reader (BMG Labtech) every 60 minutes for 5 hours. After this time Triton X-100 was added (1 % vol/vol in PBS, 1 mL) to each well and the plate incubated at room temperature for 20 minutes. Aliquots of the solutions in each well (200 μL) were then transferred into a clear 96 well plate and the fluorescence measured.

2.7 Assaying vesicle-cell interactions
Cells were seeded as a monolayer onto 24-well cell culture plate and incubated for 12 hours (cell count of 39 cells/μL, total cell count 40,000). Suspensions of vesicles with encapsulated 5/6-carboxyfluorescein (25 μL, total lipid concentration = 14 mM) were then added to: a monolayer of cells; a layer of extracellular matrix (where the cells had been removed using cell dissociation buffer); tissue culture polystyrene which had been incubated with media for 12 hours. The mixtures were then incubated for 12 hours. Aliquots of the media (0.2 mL) were transferred to a 96 well plate and the fluorescence measured using a Fluorostar optima fluorescence plate reader (BMG Labtech). The vesicle samples in the cell culture wells were then washed with PBS (10 × 1 mL), aliquots of the final wash transferred into a 96 well plate and the fluorescence measured. Finally the vesicle samples in the cell culture wells were incubated with Triton X-100 (1 % vol/vol in PBS, 1 mL) for 20 minutes at room temperature. Aliquots of the Triton X-100 solutions (0.2 mL) were transferred into a 96 well plate and the fluorescence measured.

2.8 Cell staining and imaging of cells with Bt-DPPC vesicles in media or alginate mixtures using confocal fluorescence microscopy.

Harvested cells were resuspended in fresh medium and were seeded onto glass coverslips at a density of 1 × 10^5 cm^-2. Cells were incubated with rhodamine labeled Bt-DPPC vesicles suspended in PBS or sodium alginate (total lipid concentration = 20 mM, 200 μL) at 37 °C and 5 % CO₂ (vol/vol) for 12 hours. The culture medium was removed and the samples washed with PBS (10 × 1 mL). Samples were fixed using paraformaldehyde (4 % wt/vol in PBS, 1 mL) for 15 minutes then washed with PBS. The cells were blocked using ICC block (PBS containing Triton X-100 at 1 % vol/vol, bovine serum albumin 1 % wt/vol) and stained with FITC-tagged phalloidin (0.165 µm phalloidin) in 1 % bovine serum albumin for 20 minutes. Samples were washed with PBS (5 × 1 mL) and then counterstained with DAPI and mounted using Prolong antifade (Invitrogen, UK).

2.9 Flow cytometric analysis of cells with Bt-DPPC vesicles

Cells were grown on tissue culture plastic until 60 % confluency was reached. Vesicle suspensions with or without encapsulated FITC dextran (25 μL, 14 mM) were then added to the samples and the cells cultured for a further 12 hours. Cells were harvested using cell dissociation buffer (CDB) centrifuged at 700 rpm for 3 minutes and resuspended in FACS
buffer (0.5 mL, PBS containing 2 mM EDTA and 0.5 % BSA wt/v) and stored on ice. The samples were analyzed by flow cytometry, which were carried out on a Becton Dickinson FacsCalibar using CellQuest software.

3 Results and Discussion

3.1 Stability of Bt-DPPC vesicles under cell culture conditions

The loss of encapsulated material from thermally-sensitive phospholipid vesicles occurs when the bilayers undergo a phase transition upon heating to the membrane melting temperature \( T_{m} \), corresponding to the vesicle bilayer entering a liquid disordered phase from the solid ordered phase [17]. The melting temperature is characteristic for each phospholipid bilayer and DPPC vesicles undergo this transition at 41 °C [18]. The \( T_{m} \) of DPPC bilayers should ensure stability under physiological conditions (i.e. 37 °C), yet the vesicles should still be able to release their contents thermally during culture, a key requirement of our magnetic delivery system [6]. We used Bt-DPPC vesicles with encapsulated 5/6-carboxyfluorescein (5/6-CF, a model for water-soluble bioactive compounds) and assayed membrane disruption of the vesicles under cell culture conditions (Fig. 1). Release of 5/6-CF from compromised vesicles is self-indicating, as a large increase in fluorescence results from the alleviation of self-quenching inside the vesicle. After incubation of Bt-DPPC vesicles for 5 hours at 37 °C and 5 % vol/vol CO\(_2\), no spontaneous release of 5/6-CF was observed; subsequent addition of Triton X-100 at 5 hours completely disrupted the membrane and caused full release of encapsulated material. This confirms that as expected, adhesive Bt-DPPC vesicles can successfully store compounds under typical conditions used for cell culture.

3.2 The effect of interactions with cells or surfaces on Bt-DPPC vesicle stability

As well as direct interactions with cells, adhesive Bt-DPPC vesicles could also interact with the extracellular matrix produced by proliferating cells and the surface upon which the cells sit. The interactions of Bt-DPPC vesicles with three relevant surfaces were therefore assessed: tissue culture plastic, cell-free extracellular matrix and a fibroblast monolayer. The surface of tissue culture polystyrene is plasma treated to generate a hydrophilic surface that encourages the culture of anchorage dependent cells, while the extracellular
matrix of 3T3 fibroblasts is a complex mixture of polysaccharides (such as glycosaminoglycans) and proteins that are secreted from cells. Fibroblast cells grown as a monolayer also display a range of membrane-anchored receptors which control cellular interactions, for example detecting and binding to the surroundings.

Bt-DPPC vesicles encapsulating 5/6-CF were used to monitor the adhesive interaction between the vesicles and the surfaces. The presence of intact vesicles can be revealed by the addition of Triton X-100, which lyses the vesicles to release the 5/6-CF and give a fluorescent signal. Addition of Bt-DPPC vesicles to each of the three surfaces did not release 5/6-CF, indicating that any adhesive interactions with the surfaces did not disrupt the vesicles. Each sample was then washed thoroughly with PBS to remove unbound Bt-DPPC vesicles, and Triton X-100 added to each. A significant fluorescence signal from the lysis of bound vesicles was only obtained from the sample with both Bt-DPPC vesicles and a fibroblast monolayer (Fig. 2); the fraction of Bt-DPPC vesicles that had adhered to the cells could be calculated as 3 to 4% of the number added. The addition of non-biotinylated DPPC vesicles to a fibroblast monolayer gave a smaller fluorescence signal after washing and Triton X-100 addition, indicating that the adhesive interaction was largely occurring via the biotin coating. Given there was no recognition of the extracellular matrix or tissue culture plastic, this suggests the biotin coating on the Bt-DPPC vesicles is recognized by membrane-bound biotin receptors on the fibroblasts.

The stability of these fibroblast-vesicle assemblies over a 72 hour period was then assessed. The fluorescence due 5/6-CF released from cell-bound vesicles was monitored both before and after the addition of Triton X-100. Over this period the population of cells will rapidly increase, as 3T3 fibroblasts can double their population over 120 hours even when cultured in alginate/fibronectin gels [16]. Every 24 hours the fluorescence of each of the samples was measured, followed by Triton X-100 addition to lyse the bound vesicles and release 5/6-CF. Despite ongoing cell division, Bt-DPPC vesicles attached to cells showed a high level of membrane integrity, with no release of 5/6-CF over a 72 hour period. Subsequent vesicle lysis with Triton X-100 produced a similar level of fluorescence over the 72 hour period; fluorescence measurements after Triton X-100 addition showed 2.9 ± 0.4 % of the originally added Bt-DPPC vesicles had bound to the cells after 1 hour, while after 3 days this value had only slightly declined (2.6 ± 0.4 %). This showed that although the number of cells had increased, the number of intact Bt-DPPC vesicles had remained largely constant within the cell culture wells.
3.3 Influence of matrix fluidity on the evolution of Bt-DPPC vesicle-cell interactions

These preliminary studies suggested that cell-Bt DPPC vesicle links form quickly in media, but increases in the viscosity of the environment surrounding the cultured cells would be expected to slow the rate at which these cell-vesicle interactions develop. In particular, the period over which the Bt-DPPC vesicles (or assemblies of magnetic nanoparticles and Bt-DPPC vesicles [16]) are incubated with the cell/sodium alginate mixture before gelation, which will immobilize the vesicles, should determine the number of vesicle-cell assemblies formed. Longer incubation times before curing with Ca(II) may improve the efficacy of these smart biomaterials by bringing the drug carrier and cell into proximity before magnetic release.

The Bt-DPPC vesicle membranes were doped using rhodamine-DHPE, which allowed visualization of the vesicles and contrast with cells stained with FITC-phalloidin and DAPI. Confocal fluorescence microscopy revealed that rhodamine-labeled Bt-DPPC vesicles in alginate gels that had been cured as soon as the fibroblast solutions were added exhibited very few interactions between the Bt-DPPC vesicles and cell membranes. However cells seeded into sodium alginate solution and incubated with Bt-DPPC vesicles for four hours prior to gelation showed a marked increase in the number of vesicle-cell interactions. This four hour period allowed movement of the components through the alginate matrix whilst it was still fluid enough to allow diffusion (Fig. 3 a). The effect of the viscosity of the solution was also clear, as cells seeded onto glass without sodium alginate present exhibited more vesicle-cell interactions after 4 hours than the sample with sodium alginate (Fig. 3 b), showing the anionic polysaccharide chains of alginate hinder the diffusion of vesicles and cells through the samples.

3.4 The cellular location of Bt-DPPC vesicles bound to fibroblasts

Vesicles bound to cells are known to be subsequently endocytosed or fuse with membranes [10,11], so confocal microscopy was use to establish if either of these processes had occurred in the Bt-DPPC vesicle-cell assemblies. As previously, the vesicle membranes were doped using rhodamine-DHPE to contrast with cells stained with FITC-phalloidin and DAPI. Confocal microscopy was used to image through the cell structure

with slices every 0.5 μm, which should allow the exact location of the vesicles to be ascertained.

As anticipated from the studies with 5/6-CF loaded Bt-DPPC vesicles and fibroblasts, the vesicles were not observed to have merged with the membrane of the cells and instead appeared as discrete entities (Fig. 4). The vesicles were observed in the same plane as the cells, but on the upper face of the cell membrane. The vesicles covered the top of the cytoskeleton and the nuclei yet the vesicles were not discernable inside any cell structure, which supports the hypothesis that bound Bt-DPPC vesicles neither fused nor were internalized by the cells.

3.5 Flow cytometric studies of Bt-DPPC vesicle-cell assemblies

Flow cytometry was used to ascertain if the adhesion of Bt-DPPC vesicles to 3T3 fibroblasts gave a measurable increase in size in the resulting aggregates. The Bt-DPPC vesicle/cell mixtures were monitored for changes in size and fluorescence against two control samples; cell-free vesicles and vesicle-free cells (Fig. 5). As well as mixing with blank Bt-DPPC vesicles, Bt-DPPC vesicles containing FITC-tagged dextran (4 kDa) were also mixed with 3T3 fibroblasts, enabling the vesicle-cell assemblies to be tracked fluorescently. For each of the samples, particle size and granularity was obtained from the flow cytometry data. Bt-DPPC vesicles that were not interacting with cells were smaller and less granular than cells, appearing at the low size/low granularity region in the bottom left of the plots, while 3T3 cells were observed in a higher size/higher granularity region. Density analysis of 3T3 fibroblasts cultured with empty Bt-DPPC vesicles and FITC-dextran encapsulating Bt-DPPC vesicles appeared to show three different populations compared to cells with no vesicles present, which had a tight population (Fig. 5 a-c). In addition to cell-free Bt-DPPC vesicles, density analysis showed the 3T3 fibroblasts now comprised two size-distinct cell-containing populations (marked in red, Fig. 5 b, c). The increases in the size and granularity profile of 3T3 fibroblasts support the suggestion that Bt-DPPC vesicles had bound at the cell surface (Fig. 5 d, e). The lower granularity population was slightly smaller in size and less fluorescent, suggesting the differences between the two cell-containing populations could be ascribed to the number of Bt-DPPC vesicles attached to the cells, with larger vesicle-cell aggregates appearing at higher size and granularity. Furthermore only 3T3 fibroblasts cultured with FITC-dextran containing Bt-DPPC vesicles showed green fluorescence (Fig. 5
f), confirming the adhesive link between intact vesicles and the cells. After flow cytometric analysis had been carried out, the samples were collected and imaged to ensure the vesicle structures remained intact [19]. Microscopy on the samples after flow cytometric analysis showed large variation in the number of FITC-dextran containing Bt-DPPC vesicles attached to cells, with some cells bound to large numbers of vesicles and others attached to very few vesicles.

4 Conclusions

We have demonstrated that Bt-DPPC vesicles can retain their contents in vitro under cell culture conditions and that cell surface interactions with the biotin coating can target these vesicles to cells. These interactions only occurred with the cell membranes, and were not observed between the Bt-DPPC vesicles and extracellular matrix or tissue culture plastic. These interactions between Bt-DPPC vesicles and the fibroblasts required the presence of the biotin coating around the vesicle membrane, suggesting biotin lipids can mediate vesicle attachment to cell surfaces. A number of biotin-recognizing receptors are known to be present in cell membranes, including fibroblasts, and several are overexpressed in cancerous cells [20]. Pleasingly the Bt-DPPC vesicles adhere to the fibroblasts intact, without fusion or endocytosis, which has important implications for remotely triggered drug delivery. However for this interaction to be maximized within a hydrogel cell culture scaffold, the cells and vesicles must be co-incubated for several hours before the mixture is gelled into a robust biomaterial. This stands in contrast to previous studies of N-(biotinyl)-dioleoyl phosphatidylethanolamine vesicles with mesenchymal stem cells that has indicated membrane fusion was the primary outcome, but this may have been due to high vesicle fluidity and the fusogenic nature of unsaturated dioleoyl phosphatidylethanolamine lipids used in the vesicles [11,12]. The cell-vesicle interactions demonstrated here maybe useful for further applications of vesicles in vitro and in vivo for targeted drug release and directed tissue culture. In particular we hope to use the results of these studies to improve the efficiency of remotely triggered drug delivery in our magnetically responsive alginate biomaterials [6]. Through using biotinylated magnetic nanoparticle/vesicle assemblies and incubating these assemblies with cells prior to curing of the alginate gel, we should be able to bring the cells and vesicles into proximity, increasing the local concentration of magnetically released drug around to the cultured cells. Further studies in this area are ongoing.
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References:


Fig. 1. Stability of Bt-DPPC vesicles with encapsulated 5/6-carboxyfluorescein under cell culture conditions. Triton X-100 was added at 5 hours and the fluorescence data was normalised to the maximum fluorescence observed from each sample.

Fig. 2. Release of 5/6-carboxyfluorescein (5/6-CF) from Bt-DPPC vesicles (Bt-DPPC) or blank vesicles (DPPC without biotin) that have been exposed to different surfaces, as monitored by fluorescence (background fluorescence has been subtracted). Left: after washing with PBS buffer. Right: after Triton X-100 was added to the samples.
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**Fig. 3.** Formation of Bt-DPPC vesicle/fibroblast assemblies in different matrices. (a) sodium alginate solution containing Bt-DPPC vesicles (labeled with red fluorescent rhodamine DHPE, bright spots, red in online version) which was seeded with cells and cultured for 4 hours prior to gelation with Ca(II). (b) Bt-DPPC vesicles and cells seeded onto glass and incubated in media for 4 hours. Scale bar 20 µm.

**Fig. 4.** Z-stack of confocal microscopy images showing rhodamine-DHPE tagged vesicles (bright spots, red in online version) linked to 3T3 fibroblasts stained with FITC-phalloidin and DAPI. (a) 0 µm; (b) -0.5 µm; (c) -1.0 µm; (d) -1.5 µm; (e) -2.0 µm. Scale bar 25 µm.
Fig. 5. Analysis of Bt-DPPC vesicle/fibroblast assemblies by flow cytometry. 3T3 Fibroblasts were cultured for 24 h with or without empty Bt-DPPC vesicles (control) or Bt-DPPC vesicles encapsulating FITC-dextran, and processed for flow cytometry as described in the Methods section. (a) A density plot (particle size (x-axis) vs. granularity (y-axis)) of 3T3 fibroblasts, which gate in a single population on the plot. (b) Density analysis of 3T3 cells cultured with empty Bt-DPPC vesicles and (c) FITC-Dextran encapsulating Bt-DPPC vesicles. In both cases the 3T3 fibroblasts now comprise two size-distinct populations (marked in red). Unbound Bt-DPPC vesicles are smaller and less dense than cells, thus appearing in the bottom corner of the plots. (d, e, f) Comparison of distribution profiles in each of the three conditions: dashed trace (red online) = 3T3 fibroblasts alone; black trace = 3T3 fibroblasts cultured with empty Bt-DPPC vesicles; gray trace (green online) = 3T3 fibroblasts cultured with FITC-dextran encapsulating Bt-DPPC vesicles. (d) Comparison of the size distributions. (e) Comparison of the granularity distributions. (f) Comparison of the fluorescence distributions.
Chapter 4
Conversion of Magnetic Impulses into Cellular Responses by Self-Assembled Nanoparticle–Vesicle Hydrogels
F de Cogan, JE Gough, SJ Webb
Phospholipid vesicles are widely used as nano-sized drug delivery vehicles\(^{[11]}\) and as biomimetic model systems\(^{[13]}\), where the bilayer allows fundamental biomembrane processes like ion transport, signalling and multivalent recognition to be copied\(^{[13]}\). In particular, the remotely triggered transit of stored chemicals across bilayer membranes is a key goal as it will allow vesicles to communicate with cells either in vivo or in vitro during cell culture.\(^{[14]}\) The latter approach should produce exciting new “smart” biomaterials, although non-invasive and non-chemical control over drug release from vesicles remains challenging.

Most mammalian cells are unaffected by oscillating or permanent magnetic fields. To sensitize cells to magnetic fields, they can be labeled with magnetic nanoparticles (MNsPs)\(^{[15]}\), an approach used to effect gene transfection with static magnetic fields\(^{[16]}\) or cause hyperthermia with alternating magnetic fields (AMFs).\(^{[17]}\) Alternatively MNPs can be used to label vesicles, which allows magnetic manipulation and AMF-triggered contents release; magnetic release is attractive as nearby cells would only be affected by the released biochemicals and not the AMF. Recently we used 10 nm Fe\(_2\)O\(_4\) MNPs to crosslink 800 nm diameter phospholipid vesicles and form magnetic nanoparticle-vesicle assemblies (MNPVs)\(^{[18]}\). Embedding MNPVs within a hydrogel matrix added a further level of assembly, with the hydrogel fibrils acting as an artificial extracellular matrix that reinforced the vesicles, providing robust materials\(^{[19]}\) that responded to AMFs by releasing stored dyes. Such nano-structured and responsive self-assembled biomaterials have enormous potential in cell culture,\(^{[20]}\) and replacing these dyes with bioactive species should produce a new type of “smart” cell culture scaffold\(^{[21]}\) responsive to magnetic impulses. Herein we describe a self-assembled bionanotechnological system able to act as a “smart” biomaterial that translates non-invasive magnetic signals into cellular responses (Figure 1).

An important design feature was the self-assembly of Fe\(_2\)O\(_4\) nanoparticles with gel-phase vesicles, an alternative to physical incorporation\(^{[12]}\) that was designed to allow heat generated in the MNPs by the AMF to be efficiently transferred to the bilayers.\(^{[13]}\) When heated, gel-phase vesicles “melt” at a triggering temperature \(T_m\), an all-or-nothing event that allows complete and rapid escape of encapsulated compounds. The biotin-avidin interaction was used to link vesicles and MNPs, which improved compatibility across cell types, including myoblasts (Figure 2) and chondrocytes. It also allowed commercially available biotin lipids like \(N\)-(biotinoyl)-1,2-dihexadecanoyl phosphatidylethanolamine (biotin-DHPE) to be used as vesicle crosslinkers\(^{[14]}\).

\(N\)-Biotinylated dopamine (1)\(^{[15]}\) was used to give Fe\(_2\)O\(_4\) MNPs an adhesion coating. MNPs were formed by co-precipitation\(^{[16]}\) then sonicated with 1 in deoxygenated methanol (0.7 mM) to give 1-coated Fe\(_2\)O\(_4\) nanoparticles ([1-MNP]), with a coating efficiency of 50 ± 20 % (Figure 2a). Dipalmitoyl phosphatidylcholine (DPPC) vesicles (800 nm diameter) were chosen as the nanocounters as these bilayers have \(T_m\) ~ 42 °C\(^{[17]}\), a triggering temperature above cell culture conditions (37 °C). Vesicles with stored chemical payloads were created by extrusion of 0.2 % mol/mol biotin-DHPE 2 in DPPC in a solution of the compound to be encapsulated. The thermal release of encapsulated 5/6-carboxylfluorescein (5/6-CF) showed these [2-DPPC] vesicles had \(T_m\) ~ 45 °C. Addition of the avidin “glue” to a mixture of [1-MNP] and [2-DPPC] vesicles produced large magnetic nanoparticle-vesicle assemblies (MNPVs); using fluorescein-labeled avidin with rhodamine-labeled vesicles revealed these components were co-localized in the MNPVs (Figure 2b).\(^{[18]}\) These MNPVs were susceptible to external magnetic fields,

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**Figure 1:** a) Cells and nanoparticle-vesicle assemblies (MNPVs) are co-immobilized within a calcium alginate hydrogel (yellow). MNPVs are self-assembled nanocarriers composed of magnetic nanoparticles coated with biotinoyl dopamine 1 (1-MNP) and DPPC vesicles containing biotin-DHPE 2 (2-DPPC), which are linked together by avidin. b) Chemical messengers, such as drugs (blue), can be non-invasively released by an alternating magnetic field (AMF), and these released chemicals in turn induce responses from cultured cells.

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To obtain robust biomaterials, these MNPVs were immobilized in a calcium alginate matrix. This hydrogel allows these smart biomaterials to be compatible with many cell types, particularly chondrocytes, while being easy to form and manipulate at physiological temperatures. Purified MNPVs were magnetically sedimented then re-suspended in 2 % wt/vol sodium alginate in phosphate buffered saline (PBS). The mixture was then gelled by infusion of CaCl₂ (0.1 M) through a polycarbonate membrane; adding cells prior to gelation produced materials with cells and MNPVs co-immobilized. Confocal microscopy showed cells surrounded by the smaller MNPVs (Figure 2c); adhesive interactions between cells and MNPVs occur if the incubation at 50 °C after gelation produced materials with cells and MNPVs co-immobilized. Confocal microscopy showed cells surrounded by the smaller MNPVs (Figure 2c); adhesive interactions between cells and MNPVs occur if they are co-incubated for > 4 h before gelation.

The efficiency of magnetic release was quantified using encapsulated 5/6-CF. Fluorescence spectroscopy indicated little release of 5/6-CF (0.05 M inside the vesicles) occurred during gelation and the MNPVs largely remained intact. However 5/6-CF rapidly escaped from a gel block (25 mm²) after exposure to a 392 kHz AMF pulse (240 s). The amount of 5/6-CF that escaped from the gel was quantified after 2 h, which gave 1.3 µM 5/6-CF in the solution covering the gel block (2 mL); without an AMF only 35 % escaped after 3 days. The corresponding concentration within the hydrogel after magnetic release but prior to diffusion out of the gel block was calculated as ~100 µM, which should allow biochemicals with micromolar K_d values to induce cellular responses.

Biomaterials composed of chondrocytes encased within calcium alginate have shown promise as engineered scaffolds to replace articular cartilage, with the cells producing cartilage markers such as glycosaminoglycans and collagen II. For the production of extracellular collagen by chondrocytes and osteoblasts, which normally occurs over several days, ascorbic acid is an essential additive. Therefore either ascorbate (150-300 µM) or ascorbic acid-2-phosphate (AAP, a polar and air-insensitive precursor that is readily converted to ascorbate by cells) are typically added to chondrocyte cell culture media.

The response of chondrocytes to AAP was the ideal model system to illustrate magnetically-induced changes in cellular behavior. Chondrocytes show good tolerance for calcium alginate matrices, with the inert 3D alginate matrix maintaining their rounded morphology and therefore their phenotype. Furthermore, adding MNPVs (~20 mM DPPC) did not significantly affect the growth of chondrocytes in the gel (Figure 3a).

The encapsulation and release of AAP (8.6 mM) from alginate embedded MNPVs was demonstrated in a similar manner to 5/6-CF. A pulse of AMF (240 s) released encapsulated AAP from a 25 mm² gel block, and the concentration of the AAP in the surrounding PBS solution (2 mL) was determined by phosphatase hydrolysis followed by titration with triiodide (Figure 3b). This showed that the concentration in the gel block after release was ~400 µM, which should be high enough to induce a strong cellular response. The release rate of anionic AAP at pH 7.4 was akin to that observed for 5/6-CF, with 40 % release after 0.5 h and 80 % release after 1.5 h (compared to incubation at 50 °C). This suggests that the passage of bioactive anions through calcium alginate is not significantly impeded by the hydrogel matrix.

To measure the response of chondrocytes cultured in these AAP-loaded MNPV hydrogels to an AMF, alginate gels containing chondrocytes (25,000 cells per 500 mm²) and MNPVs (~20 mM DPPC) were fabricated in glass vials, while control hydrogels containing [2-DPPC] vesicles with stored AAP but no [1-MNP] were also formed. Media (1 mL) was added to cover the gel and the samples incubated at 37 °C. Collagen production was measured at regular intervals over 14 days using the Sircol collagen assay. After an initial lag period following application of the AMF, the chondrocytes in the gel responded to the magnetic release of AAP by producing collagen at levels comparable to literature studies (up to 6 mg/mL, Figure 4a). Little cell response was observed with uncoated MNP and [2-DPPC], showing an adhesive link between both components of the MNPVs is needed to produce a cellular response to the AMF.

The production of collagen by chondrocytes in response to the magnetic signal was visualized after 14 days using fluorescence microscopy on gels containing AAP-loaded MNPVs. Little collagen was produced in samples that had not been exposed to an AMF (Figure 4b). However, large amounts of extracellular collagen I and collagen II were produced in samples exposed to an AMF, which formed a mesh around the chondrocytes (Figure 4c). This response shows the potential of these 3D cell culture scaffolds for non-invasive production of cartilage-like extracellular matrices.\(^{28}\)

Chondrocytes and added this extracellular matrix protein to the alginate scaffold. The transparency of tissue to magnetic fields means that cellular responses could be initiated non-invasively in vivo, as recently illustrated with in vivo magnetic release of cells from ferrogel-based scaffolds.\(^{29}\) Given external magnetic fields can pattern MNPVs within these materials, we hope in future to initiate cellular responses that are both spatially and temporally controlled.

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**Figure 4:** (a) AMF-induced production of collagen by chondrocytes: (A) calcium alginate only; (B) AAP-containing [2-DPPC] vesicles in calcium alginate; (C) AAP-containing [2-DPPC] vesicles with uncoated MNPVs in calcium alginate. (D) AAP-containing MNPVs in calcium alginate. (B,c) Confocal microscopy images of alginate gels containing chondrocytes and MNPVs, with fluorescent staining of cell nuclei (blue). Top: stained for collagen II (stained red with tetramethyl rhodamine isothiocyanate (TRITC)-secondary antibody). Bottom: stained for collagen I (stained green with FITC-secondary antibody).

In summary, we have developed a self-assembled and biomimetic biomaterial that responds to a magnetic signal by releasing the contents of phospholipid vesicles. Crosslinking thermally-sensitive DPPC vesicles with magnetically-responsive Fe\(_3\)O\(_4\) nanoparticles allowed remote magnetic release of the vesicle contents, which occurred at low loadings of Fe\(_3\)O\(_4\) and did not otherwise affect cells within the material. An alginate gel was the synthetic extracellular matrix around the MNPVs, producing robust materials suitable for cell culture. We found that encapsulating polar compounds at \(~10\) mM within the vesicles gave sufficient concentrations after magnetic release to generate cellular responses. The exciting potential of these materials was exemplified using magnetic release of AAP, which switched on collagen production by chondrocytes and added this extracellular matrix protein to the alginate scaffold.

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[13] Hyperthermia assays suggest release occurs through MNP heating via Néel relaxation, but membrane disruption due to MNP oscillations could also be possible.
[18] Several other assembly outcomes may also occur, including crosslinking of biotin residues across the surface of MNPs or vesicles, which could lower release efficiency.

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[27] Chondrocytes have been reported as producing 5 mg.ml⁻¹ collagen in an alginate hydrogel. See: G. M. Williams, T. J. Klein, R. L. Sah, Acta Biomater. 2005, 1, 625-633.


S.1. Materials and instrumentation

NMR spectra were measured on Bruker DPX300, AV400 or AMX500 instruments for solutions in CDCl₃ or CD₃OD, and were assigned with the aid of COSY, HMBC, HMQC, and DEPT spectra where appropriate. Coupling constants are given in Hz; multiplicities are given as singlet (s), doublet (d), triplet (t), quartet (q), quintet (qn), sextet (sxt), septet (spt) or multiplet (m), with broad signals indicated by using the abbreviation br. Electrospray mass spectra were measured on a Micromass LCT instrument using a Waters 2790 separations module with electrospray ionisation and TOF fragment detection. Samples were prepared by using a 50:50:0.1 acetonitrile/water/formic acid solution. HRMS measurements were made on a Thermo Finnegan MAT95 XP instrument. UV-Visible spectra were recorded on a Jasco V-660 spectrophotometer with the temperature controlled by a Jasco EHC-716 Peltier. Fluorescence spectra were recorded on a Perkin-Elmer LS55 fluorimeter, with temperature control using a Julabo F25-HE water circulator. Images of vesicle-nanoparticle assemblies were captured using a Zeiss Axio Imager A1 fluorescence microscope fitted with a Canon Powershot G6 digital camera. Transmission electron microscopy was carried out on a Joel 1220 TEM transmission electron microscope coupled to a high resolution GATAN ORIUS CCD camera. Induction heating was carried out with a water-cooled EASYHEAT 0224 induction heater (2.0 kW, 150-400 kHz) with an EASYHEAT 300P workhead, purchased from Cheltenham Induction Heating, Gloucestershire GL52 6RU, U.K. Neodymium iron boron (NdFeB) magnets, either N48 (5350 G, 23 mm diameter x 20 mm length x 6 mm diameter c/sunk hole) or N42 (4700 G, 3 mm diameter x 13 mm length), were purchased from e-magnets UK, Sheffield S2 5QT, U.K. Chemicals were used as received from Sigma-Aldrich except for 1 which was synthesized as detailed in S.2. below.

S.2. Synthesis and characterization of 1

Biotin-dopamine conjugate 1 was synthesized either following literature protocols¹, or prepared in smaller batches according to the synthetic scheme below.
Synthesis of \( N \)-biotinyl-(3,4-dibenzyloxyphenylethylamide) 3.

Biotin (0.115 g, \( 4.7 \times 10^{-4} \) mol), \( N,N,N',N' \)-tetramethyl-\( O-(1H \)-benzotriazol-1-yl)uronium hexafluorophosphate (0.227 g, \( 6.0 \times 10^{-4} \) mol) and di(iso-propyl)ethylamine (DIPEA, 1.25 mL) were added to dry DMF (15 mL) under dry nitrogen. The reaction was stirred for four hours at 0°C, then 3,4-dibenzyloxyphenylethylamine hydrochloride (0.120 g, \( 3.2 \times 10^{-4} \) mol) and DIPEA (1.25 mL) were added to the reaction under nitrogen and the reaction stirred at room temperature for 12 hours. The DMF was evaporated under reduced pressure and the residue was washed with H\(_2\)O (10 mL), extracted with ethyl acetate (3 \( \times \) 10 mL) and dried over MgSO\(_4\). After filtration and evaporation under reduced pressure, the residue was purified using column chromatography (6 % vol/vol methanol in CH\(_2\)Cl\(_2\)/silica gel) to give the title compound as a yellow solid (0.15 g, 84 % yield). \( ^1 \)H NMR (400 MHz, CDCl\(_3\), 25 °C): \( \delta = 1.24 - 1.34 \) (m, 2H, (C=O)CH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)), 1.44 - 1.65 (m, 4H, (C=O)CH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\) and (C=O)CH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)), 2.04 (t, \( J = 7.4 \) Hz, 2H, (C=O)CH\(_2\)CH\(_2\)), 2.58 (d, \( J = 16.0 \) Hz, 1H, S\( CH_2 \)CHNH syn to side chain), 2.63 (t, \( J = 6.9 \) Hz, 2H, CH\(_2\)CH\(_2\))NH), 2.76 (dd, \( J = 12.9, 4.9 \) Hz, 1H, S\( CH_2 \)CHNH, anti to side chain), 2.99 (td, \( J = 7.3, 4.7 \) Hz, 1H, CH\(_2\)CH\(_2\)NH(C=O)), 3.33 (q, \( J = 6.7 \) Hz, 2H, CH\(_2\)CH\(_2\)NH), 4.15 (dd, \( J = 7.6 \) Hz, 5.4 Hz, 1H, CH\(_2\)CH\(_2\)NH(C=O)), 4.33 (dd, \( J = 7.7, 5.0 \) Hz, 1H, CH\(_2\)CH\(_2\)NH(C=O)), 5.05 (s, 2H, PhCH\(_2\)), 5.07 (s, 2H, PhCH\(_2\)), 5.88 (t, \( J = 5.7 \) Hz, 1H, CH\(_2\)CH\(_2\)NH), 6.21 (s, 1H, CH\(_2\)CH\(_2\)NH(C=O)), 6.63 (dd, \( J = 8.2, 2.0 \) Hz, 1H, OC\(_2\)CH\(_2\)), 6.73 (d, \( J = 2.0 \) Hz, 1H, OC\(_2\)CH), 6.81 (d, \( J = 8.2 \) Hz, 1H, C\(_2\)CH\(_2\)CO), 7.17 - 7.41 (m, 10H, CH\(_2\)Ph). Note: integration and COSY data indicates an amide NH (biotin) signal is underneath the signals 5.07-5.04. \( ^{13} \)C NMR (100 MHz, CDCl\(_3\), 25 °C): \( \delta = 25.40 \) (CH\(_2\)CHS), 27.88 (C(O)CH\(_2\)CH\(_2\)), 28.21 (CH\(_2\)), 35.06 (C(O)CH\(_2\)), 36.02 (Ar-CH\(_2\)CH\(_2\)), 40.31 (S\( CH_2 \)), 40.74 (CH\(_2\)NH), 55.39 (CH\(_2\)CHS), 60.41 (CH\(_2\)SH), 62.03 (NHCH\(_2\)SH), 71.15...
Synthesis of N-biotinyl-(3,4-dihydroxyphenylethylamide) 1.

N-Biotinyl-(3,4-dibenzoxyphenylethylamide) 3 (0.030 g, 5.36 × 10⁻⁵ mol) was dissolved in 5:1 MeOH:CH₂Cl₂ (5 mL). This solution was cycled through a Thales Nanotechnology H-Cube model HC-2 using a 20% Pd(OH)₂/C catalyst (CatCart 30) at 1 mL/minute, 30°C and under 70 bar of H₂ until the starting material was no longer visible by TLC (approx. 2 h). The system was flushed with MeOH (10 mL) before and after the procedure, the latter flush was added to the product solution and the solvent was removed under reduced pressure to yield N-biotinyl-(3,4-dibenzoxyphenylethylamide) 1 (0.016 g, 4.16 × 10⁻⁵ mol, 78% yield) as a clear, colorless oil. This material gave spectral data consistent with previously published data.\[1\] ¹H NMR (400 MHz, CD₃OD, 25 °C): δ = 1.27 – 1.42 (m, 2H, (C=O)CH₂CH₂CH₂). 1.44 – 1.78 (m, 4H, (C=O)CH₂CH₂ and (C=O)CH₂CH₂CH₂CH₂), 2.18 (t, J = 7.3 Hz, 1H, NH(C=O)CH₂CH₂), 2.65 (t, 2H, J = 6.9 Hz, CH₂CH₂NH(C=O)), 2.72 (d, J = 12.7 Hz, 1H, d, J = 16.0 Hz, 1H, S(CH₂CHNH syn to side chain), 2.96 (dd, J = 12.8, 5.0 Hz, 1H, SCH₂CHNH, 9

Figure S1: ¹H NMR of compound 3.
anti to side chain), 3.18 (dt, J = 9.1 Hz, 5.5, 1H, CH$_2$CHNH(C=O)), 3.33 (dt, J=3.1, 1.6 Hz, 2H, CH$_2$NH(C=O)), 4.27 – 4.35 (m, 1H, CHCHNH(C=O)), 4.48 - 4.58 (m, 1H, CH$_2$CHNH(C=O)), 6.55 (dd, J = 8.0, 2.0 Hz, 1H, OCCHCHC), 6.66 (d, J = 2.0 Hz, 1H, OCCHC), 6.70 (d, J = 8.0 Hz, 1H, CCHCHCO). $^{13}$C NMR (100 MHz, CD$_3$OD, 25 °C): δ = 26.94 (CH$_2$CHS), 29.43 (C(O)CH$_2$CH$_2$), 29.59 (C(O)CH$_2$CH$_2$CH$_2$), 35.81 (C(O)CH$_2$), 36.78 (Ar-CH$_2$CH$_2$), 41.05 (SCH$_2$), 42.03 (CH$_3$NH), 56.93 (CH$_2$CHS), 61.76 (NHCHCHS), 63.43 (NHCHCH$_2$S), 116.33 (Ar OCCHCH), 116.91 (Ar CH), 121.07 (Ar OCCHCH), 131.96 (Ar C), 144.75 (Ar C), 146.21 (Ar C), 165.47 (NHCO(NH), 175.90 (NHCOCH$_2$). MS (ES+): m/z 380.4 [M + H]$^+$

Figure S2: $^1$H NMR of compound 1.

S.3. Synthesis and characterization of 1-MNP

**Synthesis of 10 nm diameter Fe$_3$O$_4$ magnetic nanoparticles (MNP)**

Iron (II) chloride tetrahydrate (0.429 g, 2.16 mmol) and iron (III) chloride hexahydrate (1.168 g, 4.32 mmol) were dissolved in deoxygenated water (5 mL). Sodium hydroxide solution (1 M, 20 mL) was added dropwise to the solution and the mixture stirred under nitrogen for 30 minutes. The Fe$_3$O$_4$ magnetic nanoparticles were sedimented using neodymium iron boron magnet (NdFeB, 5350 G) and the supernatant removed by decantation. The particles were washed with deoxygenated water (5 mL) then resuspended in deoxygenated water (5 mL).

This procedure was repeated five times, but for the final iteration the nanoparticles were dissolved in deoxygenated methanol and the solvent removed under reduced pressure. This gave MNP as a black powder which was stored under nitrogen (0.47 g, 2.0 mmol, 90%).

**TEM:** Samples were analyzed on a TEM microscope Joel 1220. MNPs (1.2 mg) were added to distilled water (2 mL) and the mixture sonicated for 2 h at 25 °C to create a suspension. The suspension was then spotted onto carbon coated copper grids without staining.

![TEM images of uncoated MNPs](image)

**Figure S3:** TEM images of uncoated MNPs

**DLS:** Dynamic light scattering was carried out on a Wyatt Technologies DynaPro DLS instrument. MNPs (1.2 mg, 5.0 µmol) were added to distilled water (1 mL) and the 1.2 mg/mL mixture was sonicated for 2 hours at 25 °C to create a suspension. The suspension was diluted using distilled water to give a 6.2 x 10^{-7} mg/mL suspension (5 mL). Analysis gave an average (12.5 ± 1.5) nm diameter particle size.

**Measurement of specific absorption rate (SAR):** Uncoated magnetic nanoparticles (Fe₃O₄, 6.5 mg) were suspended in distilled water (4 mL) in a glass vial (internal diameter 12 mm) and sonicated (10 minutes) to create a suspension. The vial was fitted with an insulating expanded plastic jacket and sealed with a spirit thermometer placed in the suspension. The vessel was placed within the coils of the induction heater and the change in temperature recorded during exposure to a 300 s AMF pulse (392 kHz frequency AMF at a field strength of ~42 kA/m – 2 turn solenoid, n = 140 turns/m and I = 300 A).

![Change in temperature for MNPs in distilled water (6.5 mg in 4 mL, 1.6 mg/mL) during exposure to an AMF (300 s duration). Background eddy heating has been subtracted.](image)

**Figure S4:** Change in temperature for MNPs in distilled water (6.5 mg in 4 mL, 1.6 mg/mL) during exposure to an AMF (300 s duration). Background eddy heating has been subtracted.
The SAR for uncoated nanoparticles was calculated according to the method of Drake \textit{et al}\textsuperscript{[a]} and the SAR values were corrected for eddy heating of water at 400 kHz, to give an SAR value of \((44 \pm 1) \text{ W/g (Fe}_3\text{O}_4\text{).}\)\textsuperscript{[a]} Other batches with low SAR values of \((12.0 \pm 0.1) \text{ W/g (Fe}_3\text{O}_4\text{)}\) were found to be less effective at magnetic release.

\textbf{Synthesis of 1-coated magnetic nanoparticles (1-MNP)}

Magnetic nanoparticles (250 mg, 1.08 mmol) were suspended in deoxygenated methanol (5 mL) and the biotin-dopamine conjugate 1 (60 mg, 0.158 mmol) was added. The black suspension was sonicated for 3 hours under nitrogen. The coated nanoparticles were magnetically sedimented using an NdFeB magnet (5350 G) and the supernatant removed by decantation. The particles were washed with deoxygenated water (5 mL) then resuspended deoxygenated water (5 mL). This procedure was repeated 10 times to remove any non-adsorbed biotin-dopamine, but for the final iteration the nanoparticles were dissolved in deoxygenated methanol and the solvent removed under reduced pressure. This gave 1-MNP as a black powder which was stored under nitrogen (0.21 g, 68 %). \textbf{TEM:} Samples were analyzed on a TEM microscope Joel 1220. 1-MNPs (1.2 mg) were added to distilled water (2 mL) and the mixture sonicated for 2 hours at 25 °C to create a suspension. The suspension was then spotted onto carbon coated copper grids without staining.

\textit{Figure S5:} TEM image of 1-MNPs

\textbf{DLS:} Coated 1-MNPs aggregated in aqueous suspension, which prevented accurate determination of the average particle diameter.

\textbf{Elemental analysis:} Found: C 4.34, H 0.56, N 1.07, Fe 56.41, S 0.48; Calculated for C\textsubscript{18}H\textsubscript{23}N\textsubscript{3}O\textsubscript{4}.15Fe\textsubscript{3}O\textsubscript{4}.9NaCl: C 4.89, H 0.52, N 0.95, Fe 56.82, S 0.73

\textbf{Coating efficiency from elemental analysis:} Compound 1 is 57.5 % wt/wt C while 1-MNP have 4.89 % wt/wt C. Therefore 1-MNPs are 8.50% wt/wt 1, or 0.224 µmol/mg of 1-MNP. This is equivalent to \(3.06 \times 10^{-19}\) g/particle. Since the molecular weight of the coating

Biotinylated ligand 1 is 380 g/mol, there are $8.06 \times 10^{-22}$ mol of 1 per particle. If each biotinylated ligand 1 is estimated to have a radius of rotation on the surface of $(3.5 \pm 0.5) \times 10^{-10}$ m, then 1 will occupy $(4 \pm 1) \times 10^{-19}$ m$^2$/molecule (similar to a phospholipid molecule, which occupies $7 \times 10^{-19}$ in close packed bilayer$^n$) or $(2.3 \pm 0.5) \times 10^5$ m$^2$/mol. Thus 1 covers $(1.9 \pm 0.5) \times 10^{-16}$ m$^2$/particle.

If each particle has an average radius of $r = 5.5 \times 10^{-9}$ m, then for each particle the surface area = $3.80 \times 10^{-16}$ m$^2$/particle, so the coating of 1 covers around $(50 \pm 20)$ % of the total available surface area on a particle.

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**S.4. Synthesis and characterization of [2-DPPC] vesicles**

DPPC (14.6 mg, $1.9 \times 10^{-5}$ mol) and Bt-DHPE (200 L of a 0.2 mM solution in chloroform, $4 \times 10^{-8}$ mol, 0.2 % mol/mol) were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. The lipid film was dried under vacuum for 60 minutes. The film was then hydrated in either PBS solution (1 mL), 5/6-carboxyfluorescein solution (1 mL, 50 mM in MOPS) or ascorbic acid-2-phosphate solution (1 mL, 8.6 mM in PBS). These mixtures were heated to 50°C and vortex mixed until the films were entirely resuspended. The suspensions were each extruded at a temperature above the $T_m$ through a single polycarbonate membrane (800 nm diameter pores, 19 ×) using an Avestin Liposofast handheld extruder.

**Thermal release of 5/6-CF from [2-DPPC] vesicles:** [2-DPPC] vesicles containing 5/6-carboxyfluorescein solution (1 mL, 50 mM in MOPS) were created through extrusion as detailed above. The [2-DPPC] vesicle solution (1 mL) was diluted to 2.5 mL with PBS buffer and loaded onto a pre-equilibrated PD-10 Sephadex desalting column. The vesicle solution was then eluted using a further 3.5 mL of PBS buffer. Aliquots (10 µL) of the resulting suspension were added to PBS buffer (1.99 mL) in fluorescence cuvettes. These cuvettes were equilibrated in a heated sample block at 25 °C before warming to 45 °C. The changes in 5/6-CF fluorescence emission were recorded with increasing temperature.
S.5. Synthesis and characterization of MNPVs

The vesicle suspensions (1 mL, $4 \times 10^{-8}$ mol Bt-DHPE) were added to 1-MNP coated magnetic nanoparticles (200 L of a 1.2 mg/mL suspension in PBS, 0.24 mg or $5.38 \times 10^{-8}$ mol 1) and avidin (or FITC-avidin as appropriate, 20 L of 10 mg/mL in PBS, $2.94 \times 10^{-9}$ mol avidin, $1.18 \times 10^{-8}$ mol binding sites). The mixture was left to aggregate for 60 minutes. The MNPV suspensions were then magnetically sedimented using an NdFeB magnet (5350 G) until a compact plug of magnetic material had formed at the bottom of the vial and the supernatant solution was visually free of turbidity. As much of the supernatant was removed as possible without disturbing the MNPV plug (typically 60% of the volume), and replaced with an equal volume of the appropriate buffer solution. Brief vortex mixing regenerated the vesicle suspension. This procedure was repeated at least 6 times and until the concentration of unencapsulated material was < 0.5% of the initial concentration.

After the last iteration the MNPVs were resuspended in PBS buffer (to make up to 1 mL) or sodium alginate solution (to make up to 1 mL, 2 % wt/vol in PBS, sodium alginate was autoclaved at 120 °C prior to use). These MNPVs were characterized visually by epi-fluorescence spectroscopy (Figure 2b in the manuscript text) using a Zeiss Axio Imager A1 fluorescence microscope fitted with a Canon Powershot G6 digital camera as reported previously.[7]

S.6. Synthesis and characterization of MNPV-alginate gels

MNPV/sodium alginate suspension (125 L) was added to a 25 × 5 × 1 mm trough constructed on a glass slide. The trough was covered with a polycarbonate membrane (50 nm pore size). Calcium chloride solution (0.1 M in water, 500 µL) was carefully added over

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Figure S6: Release of 5/6-CF from [2-DPPC] vesicles with increasing temperature.
the top of the membrane and the samples were incubated at room temperature for 30 minutes. After this period the MNPV/calcium alginate gel strip was cut into $5 \times 5 \times 1$ mm blocks and washed with PBS (10 mL).

**Fluorescence spectroscopy.** Thin MNPV/alginate films were prepared by adding a mixtures of MNPVs in sodium alginate (50 µL, prepared as above) to a glass slide. The alginate was cured by infusion of CaCl$_2$ solution in distilled water (0.1 M, 50 µL) through a polycarbonate membrane (50 nm pore size). The films were then observed using a Zeiss Axio Imager A1 fluorescence microscope fitted with a Canon Powershot G6 digital camera (Figure 2c in the manuscript text).

**Quantification of AMF-induced 5/6-carboxyfluorescein (5/6-CF) release from MNPV-alginate gels:** An MNPV-alginate gel block measuring 25 mm$^3$ ($5 \times 5 \times 1$ mm) was prepared as described above and placed in a fluorescence cuvette with PBS (2 mL). The cuvette was then exposed to an AMF (392 kHz) for 240 s, then the cuvette transferred to a Perkin-Elmer LS55 fluorimeter. The fluorescence emission of released 5/6-CF was monitored at 517 nm (after excitation at 492 nm). The concentration was determined by interpolation into a standard curve. Decreasing the concentration of [1-MNP] to 0.12 mg/mL of gel decreased the amount of 5/6-CF released by 75%, and it is likely that there is a lower limit for the amount of [1-MNP] needed to give AMF induced 5/6-CF release.

**Quantification of 5/6-carboxyfluorescein (5/6-CF) release from MNPV-alginate gels over time:**

Three MNPV-alginate gel blocks measuring 25 mm$^3$ ($5 \times 5 \times 1$ mm) were prepared as described above and placed in screw-capped glass vials with PBS (2 mL). An additional sample was created with MNPV-sodium alginate suspension (before gelation, 50 µL) added to PBS (2 mL). One of the cuvettes containing a gel block was then exposed to an AMF (392 kHz) for 240 s, then incubated at 37 °C. The two other samples were then incubated at 20 °C and 37 °C without AMF exposure. The fluorescence emission of released 5/6-CF in each of the samples was monitored at 517 nm (after excitation at 492 nm). The fraction of 5/6-CF released was normalized to the maximum fluorescence obtained after the addition of Triton X-100 (10 µL of 10 % v/v) and sonication (120 s).
Figure S7: Fraction of 5/6-CF released from MNPVs in sodium alginate solution at 37 °C ( ), from MNPVs in calcium alginate hydrogel at 20 °C (○); from MNPVs in calcium alginate hydrogel at 37 °C (△) and at 37 °C after exposure to an AMF (□). Curve fits are to guide the eye.

**AMF-induced 5/6-carboxyfluorescein (5/6-CF) release from [2-DPPC] mixed with [1-MNP] in calcium alginate hydrogel (without avidin “glue”).**

To remove unencapsulated 5/6-carboxyfluorescein from non-magnetic [2-DPPC] vesicles, the vesicle suspensions were purified by gel permeation chromatography (GPC as per S.4, dilution of 1 mL to 3.5 mL). The suspension obtained after GPC was re-concentrated using centrifugation (1200 rpm for 10 minutes; test runs on 5/6-CF containing DPPC vesicles showed this treatment did not cause vesicle rupture). The supernatant was removed and the vesicle plug resuspended in sodium alginate solution (1 mL in PBS, 2 % wt/vol, sodium alginate was autoclaved at 120 °C prior to use) with 1-MNP coated magnetic nanoparticles (200 L of a 1.2 mg/mL suspension in PBS) to give a solution that was ~20 mM in lipid. This mix of [1-MNP] and [2-DPPC] in suspension (125 L) was added to a 25 x 5 x 1 mm trough constructed on a glass slide. The trough was covered with a polycarbonate membrane (50 nm pore size). Calcium chloride solution (0.1 M in water, 500 µL) was carefully added over the top of the membrane and the samples were incubated at room temperature for 30 minutes. After this period the MNPV/calcium alginate gel strip was cut into 5 x 5 x 1 mm blocks and washed with PBS (10 mL). The samples were then exposed to an AMF pulse (3 min).
Figure S8: Fraction of 5/6-CF released after exposure to an AMF from [2-DPPC] vesicles mixed with [1-MNP] but without avidin in a calcium alginate hydrogel at 20 °C.

Quantification of AMF-induced ascorbic acid-2-phosphate (AAP) release from MNPV-alginate gels: Analysis was carried out using a modification of the published method of Kazakeviciene et al.[v] AAP containing MNPV-alginate gel blocks (25 mm³) were added to glass vials and suspended in PBS (2 mL). The samples were subjected to a 4 minute alternating magnetic field (AMF) pulse at 392 kHz and then incubated at room temperature. At each timepoint, aliquots (200 µL) were removed, from which smaller aliquots (3 × 50 µL) were taken and transferred to a 96 well plate (in 3 separate wells). Then alkaline phosphatase (from bovine intestinal mucosa, lyophilized powder, 2,000 – 4,000 DEA units/mg protein, 979 units/mg solid) in PBS (0.09 nM, 50 µL) was added to each well and the plate incubated at 37 °C for 30 minutes. Starch-triiodide solution (~20 µM I₃⁻ in PBS, 50 µL) was added to each well, the absorbance measured at 562 nm. A standard curve was created by measuring the decrease in the same I₃⁻/starch absorbance after the addition of known concentrations of AAP that had been incubated with alkaline phosphatase. The absorbance from each of the gel samples was interpolated into the standard curve to give the concentration of released AAP. These assays were performed in triplicate.
Figure S9: a) Standard curve for the analysis of ascorbic acid-2-phosphate (AAP) by the method of Kazakeviciene et al.\cite{6} b) Decrease in starch/triiodide absorbance after ascorbic acid-2-phosphate (AAP) release from MNPVs immobilized in alginate at (○) 20°C after 4 min exposure to 50°C water bath (△) 20°C (○) at 20°C after exposure to an AMF.

S.7. Synthesis and characterization of chondrocyte containing MNPV-alginate gels

Aliquots of sodium alginate solution that contained MNPVs with encapsulated ascorbic acid-2-phosphate (0.5 mL) were transferred into glass vials and sterilised under UV light for 1 hour. A chondrocyte cell monolayer at 70% confluency was separated using trypsin/EDTA solution (0.25 % solution, 3 mL; composed of 2.5 g/L of trypsin, 0.38 g/L of Na₄EDTA, Phenol red, Hanks balanced salt solution without CaCl₂, MgCl₂.6H₂O and MgSO₄.7H₂O), 3 mL) for 5 minutes at 37°C and fresh media (Invitrogen DMEM:F12 (1:1 – Dubelco’s Modified Eagle media: Nutrient mixture (Ham) with 10 % fetal bovine serum and 1 % penicillin streptomycin, 2 mL) added. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant removed. The cell pellet was resuspended in fresh media (5 mL) and an aliquot (20 L) transferred into a haemocytometer to ascertain a cell count.

Aliquots of sodium alginate solution containing MNPVs (0.5 mL) were then seeded with 25,000 cells added to each aliquot (25,000 cells in 0.5 mL, 50 cells/mm³). The samples prepared had chondrocytes mixed with:

- MNPVs encapsulating ascorbic acid-2-phosphate in sodium alginate. After gelation with Ca(II) (as above), the gel block (500 mm³) was suspended in media (1 mL) without ascorbic acid-2-phosphate present. Samples were either exposed to an AMF or incubated in the absence of an AMF.

- [2-DPPC] encapsulating ascorbic acid-2-phosphate in sodium alginate. After gelation with Ca(II) (as above), the gel block (500 mm$^3$) was suspended in media (1 mL) without ascorbic acid-2-phosphate present. Samples were exposed to an AMF pulse (3 min).
- [2-DPPC] encapsulating ascorbic acid-2-phosphate and uncoated MNPs in sodium alginate. To remove unencapsulated material from these non-magnetic vesicles, the vesicle suspension was purified by gel permeation chromatography (GPC, as per S.4). The suspension obtained after GPC was concentrated using centrifugation (1200 rpm for 10 minutes; test runs on 5/6-CF containing DPPC vesicles showed this treatment did not cause vesicle rupture), enough supernatant was removed to give a solution that was 20 mM lipid after resuspension in sodium alginate solution. After gelation with Ca(II) (as above), the gel block (500 mm$^3$) was suspended in media (1 mL) without ascorbic acid-2-phosphate present. Samples were exposed to an AMF pulse (3 min).
- Calcium alginate only. After gelation with Ca(II) (as above), the resulting gel block was suspended in media (1 mL) with and without ascorbic acid-2-phosphate present (173 mM). Samples were exposed to an AMF pulse (3 min).

S.8. Collagen production in chondrocyte containing MNPV-alginate gels

Prior to cell assays using chondrocytes, the effect of the AMF on the proliferation of 3T3 fibroblasts was assessed. Falcon tubes containing equal numbers of 3T3 fibroblasts were either subjected to an AMF, left outside the AMF coils for the same duration or left in the cell culture incubator continuously. Subsequent cell counting on the falcon tubes showed no statistical difference in the number of living cells in each tube after exposure to these different conditions.

As detailed in S.7, the samples to be subjected to magnetic release were placed in an alternating magnetic field for a 3 minute pulse at 392 kHz. Afterwards the samples were incubated at 37 °C with 5 % CO$_2$. At 3, 7, 10 and 14 days, samples to be analyzed for the respective time points were removed from the incubator and placed at -80 °C for at least 48 hours. The samples were then warmed to 25 °C and the media removed. Pepsin (0.1 mg/mL, 1 mL) in acetic acid (0.5 M, pH 3) was added to each sample followed by incubation at 4°C for 12 hours. Aliquots of the samples (50 L) were then transferred into Eppendorf tubes and sterile water (50 L) was added to each. Sircol dye reagent (1 mL) was added to each sample and the tubes shaken for 30 minutes at 25 °C. A standard curve for collagen was obtained by adding 5 L, 10 L, 25 L and 50 L of Sircol collagen standard (1 mg/mL collagen) to Eppendorf tubes and making up to 100 L with sterile water. Sircol dye reagent
(1 mL) was added to each standard and shaken for 30 minutes at 25 °C. The samples and standards were then microcentrifuged at 10,000 × g for 10 minutes. The solutions were then removed from each Eppendorf tube and the pellets in the Eppendorf tube dried. Sircol alkali reagent (1 mL) was added to each tube and vortex mixed for 10 minutes to dissolve the pellets in the reagent. The samples were then added to a 96 well clear bottom well plate in three aliquots (200 μL each) from each sample and the absorbance measured at 540 nm. The concentration was determined by interpolation into a standard curve. The assay was performed in triplicate.

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vi B. Kazakeviciene, G. Valincius, M. Kazemekaite, V. Razumas, Electroanalysis, 2008, 20, 2235
Chapter 5
Magnetically Triggered Drug Delivery
F de Cogan, JE Gough, SJ Webb

This chapter includes unpublished material on the release of nickel chloride and other materials for vesicle gels. A slightly altered version of this chapter has recently been submitted for publication.
Chapter 5 Magnetically Triggered Drug Delivery

Magnetically Triggered Drug Delivery
Felicity de Cogan, Simon Webb and Julie Gough

Abstract
Current treatment for cancer and other complex diseases relies on invasive surgery or drugs, which can have appalling side effects. These side effects are seen as the drugs attack healthy cells as well as tumours and infections. There is no general method to target drugs to specific areas of the body, as each drug mechanism is unique and needs to travel through different biological pathways to reach its desired location. This research builds on liposome and nanoparticle chemistry to build a novel delivery method for carrying drugs and active compounds to specific locations in mammalian anatomy. The drugs are then released using alternating magnetic fields in specific locations.

Introduction
Although recent advances in cancer chemotherapy and diagnostics have been numerous, a generic, targeted treatment for cancer still remains a challenge. As more than 1 in 3 people in the UK will develop some form of cancer during their lifetime, it is a challenge that needs to be met speedily. Most initial cancer treatments relied on the intravenous delivery of toxic drugs. Liposomal based delivery has begun to improve targeting methods with clinically approved liposome encapsulated drugs such as Doxil and Caelyx, which increase tumour uptake and decrease side effects in specific targets. However, a generic targeting method giving delivery direct to a tumour has yet to be achieved. Current targeting work is based on linking antibodies or other easily recognised biomolecules to particular areas of the body, although this has increased targeted delivery it has made each new discovery more specific to a particular cancer. Encapsulated drugs have been shown to be far more effective than unencapsulated, and lipid vesicles have been shown to be an innovative and easily modifiable method of delivery. Lipid vesicles are small artificial nanocarriers, composed of a spherical phospholipid bilayer enclosing an aqueous core. These liposomes are often formed from synthetic lipids such as DPPC. The structure and size of the vesicles is dependent on formation conditions ranging from 50 nm to > 1 μm with both uni and multilamellar structures formed. Among novel drug delivery methods, phospholipid vesicles have generated large amounts of interest due to the biocompatibility, size, low toxicity and the hydrophobic and hydrophilic properties they display. Liposomes have also been modified easily to give the required properties for targets both in vitro and in vivo. ‘Plain’ untargeted phospholipid vesicles have been used to encapsulate hydrophilic molecules in the aqueous core and hydrophobic molecules embedded in the phospholipid bilayer. These liposomes have then been targeted to the cells by decorating the phospholipid bilayer with target groups, such as folate, which targeted the folate receptor. The folate receptor is over expressed in many tumours. Incorporating stimuli sensitive
Chapter 5 Magnetically Triggered Drug Delivery

material such as Fe$_3$O$_4$ nanoparticles into the vesicles, creates magnetic vesicles or ‘magnetoliposomes’. The encapsulated magnetic nanoparticles allowed the vesicles to be positioned magnetically and have also been approved as drug delivery vehicles. The magnetic nanoparticles generally have sizes of 5-20 nm and have extensive uses in other biomedical applications, such as tumour reduction through hyperthermia and contrast imaging. It has been shown previously that by using liposomal formations and magnetic positioning, drugs have been delivered to areas where drug accumulation is traditionally low. When liposomes with encapsulated material have been targeted to the specific location, a rise in temperature has been used to release the drugs. This thermal increase has been achieved by utilising the magnetic nanoparticles. In the presence of an alternating magnetic field, the release from the liposomes was obtained due to localised heat, generated as the nanoparticles constantly realigned with the changing field (Figure 6.1).

This localised heat has also been used as hyperthermia, to selectively kill cell tissue, such as cancer tumors, in conjunction with drug release. Most cancer drugs initiate cell death either through inhibition of DNA synthesis, eg Methotrexate, or the intercalation and degradation of the DNA, eg Doxorubicin. Although not used as a drug nickel chloride is highly cytotoxic and is known to disrupt DNA structure. The exact cause of nickel triggered cell death is unknown, but it is thought to be connected to the generation of reactive oxygen species. This apoptotic process is similar to most clinically approved cancer drugs and can be used as a cheap, readily available model targeted cell death using magnetoliposomes.

Webb et al recently developed a novel formation method for magnetoliposomes. Bioactive molecules were encapsulated in thermosensitive phospholipid vesicles, which were then aggregated with Fe$_3$O$_4$ superparamagnetic nanoparticles. Both vesicles and nanoparticles had an external surface layer of biotin, which allowed aggregation to occur in the presence of avidin (Chapter 5). Herein we report the use of this system to create ‘smart’ hydrogels, which can be patterned with vesicle-nanoparticle aggregates and can interact with cells. An inductive coil is used to selectively release the cytotoxic nickel chloride from the vesicles by producing an alternating magnetic field. Ni (II) release from vesicle gels and interactions between the cells and the vesicle gel matrix were monitored. This novel system can be used to represent a drug delivery system of toxic drugs within a three dimensional in vivo environment.

2. Materials and Methods

2.1 Materials

DMPC, DPPC, biotin tagged DPPE and Rhodamine tagged DHPE were obtained from Avanti Lipids USA.
Sodium alginate, 5/6-carboxyfluroescein, calcium chloride, 3-(N-morpholino) propanesulfonic acid, DMSO, Trypan Blue, resazurin sodium salt ‘alamar blue’,
bovine serum albumin (BSA), fluorescein isothiocyanate conjugated phalloidin (FITC-phalloidin), glutaraldehyde, dimethyldialdehyde diphenyltetrazolium bromide (MTT), paraformaldehyde (PFA), diaminophenylindole (DAPI), Triton X-100, fluorescein isothiocyanate conjugated dextran (FITC-Dextran) nickel chloride, Calcein Blue and EDTA were all purchased from Sigma Aldrich, UK. L-Glutamine, penicillin/streptomycin, fetal bovine serum (FBS), trypsin-EDTA preparation, phosphate buffered saline (PBS) and Dulbecco’s modified Eagle’s medium (DMEM) F12 media were all obtained from PAA, Yeovil, UK. Tissue culture flasks and multiwall plates, ProLong Gold antifade reagent, Live/Dead assay, were all purchased from Invitrogen, Paisley, UK. 3T3 cells were obtained from the ECACC.

2.2 Formation of rhodamine vesicle aggregates.

DPPC (14.55 mg, 1.9 x 10^-5 mol) and biotinylated-DHPE (0.2 % mol/mol) were dissolved in a chloroform solution of rhodamine tagged DHPE (0.2 mM, 1 mL to give 0.1 % mol/mol). The solvent was removed in vacuo and the lipid film dried under vacuum for 60 minutes. The film was then resuspended in PBS (1 mL) and the flask heated to 50 °C and vortex mixed until the film was resuspended. The lipid mixture was then extruded 19 times through an 800 nm pore membrane. The vesicle suspension was then added to coated magnetic nanoparticles (200 µL, 1.2 mg/mL) and avidin in PBS, (50 µL, 10 mg/mL). The mixture was then aggregated for 60 minutes and magnetically purified using a NdFe magnet.

2.3 Formation of 5/6-carboxyfluorescein vesicle aggregates

DPPC (14.55 mg, 1.9 x 10^-5 mol) and biotinylated-DHPE (0.2 % mol/mol) were dissolved in chloroform (1 mL). The solvent was removed in vacuo and the lipid film dried under vacuum for 60 minutes. The film was then resuspended in 5/6-carboxyfluorescein (1 mL, 0.05 M) in 3-(N-morpholino) propanesulfonic acid (MOPS) and the flask heated to 50 °C and vortex mixed until the film was resuspended. The lipid mixture was then extruded 19 times through an 800 nm pore membrane. The vesicle suspension was added to coated magnetic nanoparticles (200 µL, 1.2 mg/mL) and avidin in PBS, (50 µL, 10 mg/mL). The mixture was then aggregated for 60 minutes and magnetically purified using NdFe magnet by magnetically sedimenting the assemblies and removing the surrounding suspension. The assemblies were then resuspended in PBS, stirred and magnetically sedimented again. This process was repeated 5 times.

2.4 Formation of nickel chloride containing vesicle aggregates

DPPC (14.55 mg, 1.9 x 10^-5 mol) and biotinylated-DHPE (0.2 % mol/mol) were dissolved in chloroform (1 mL). The solvent was removed in vacuo and the lipid film dried under vacuum for 60 minutes. The film was then resuspended in nickel chloride (7.7 mM for release studies and 15.4 mM for cell studies) in PBS (1 mL)
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the flask heated to 50 °C and vortex mixed until the film was resuspended. The lipid mixture was then extruded 19 times through an 800 nm pore membrane. The vesicle suspension was added to coated magnetic nanoparticles (200 µL, 1.2 mg/mL) and avidin, (50 µL, 10 mg/mL). The mixture was aggregated for 60 minutes and magnetically purified using NdBF₆ magnet as previously described.

2.5 Formation of vesicle gels
Alginate gel matrix was formed from (2 % wt/vol) of sodium alginate in PBS, stirred for 60 minutes and autoclaved to ensure sterility. Fibronectin from bovine plasma (2.27x10⁻⁹ M) was added to enhance cell attachment. The magnetically purified vesicle-nanoparticle aggregates were then suspended in alginate gel solution (1 mL). Calcium chloride (0.1 M, 1 mL) was added to the gel solution and the mixture incubated at room temperature for 30 minutes. The excess calcium chloride was removed and the gel block was washed with PBS.

2.6 Rheometrical studies of vesicle gels
Rhodamine tagged magnetoliposomes were made as previously described (Section 2.2). The vesicle-nanoparticle aggregates were resuspended in alginate (1 mL, 2 % wt/vol). The vesicle gel solution (0.5 mL) was added to a 24 well plate. Calcium chloride (0.5 mL, 0.1 M) was added to the well and the gel left to cure for 30 minutes. The gels were then suspended in cell media (1 mL) or PBS (1 mL) and incubated at room temperature or 37 °C for 3, 7, 10, 14 days. Gels were also suspended in media/CaCl₂ (0.005 M) at room temperature and PBS/CaCl₂ (0.005 M) at room temperature. At each timepoint the solution was removed from the around the gel block and the gel block was placed on the rheometer plate. Rheology was then carried out using cone and plate. A strain sweep was run from 0.01 to 1 % at 25 °C at a frequency of 1 Hz. Frequency sweeps were carried out from 0.01 to 100 Hz at 1 % strain. Rheometrical studies with cells present followed the same procedure until the gel solution was added to the culture plate; 3T3 fibroblast cells were then added at 10,000 cells/gel and the gel was then cured using calcium chloride (0.5 mL, 0.1 M). The gel blocks were then suspended in fresh media (1 mL) and incubated at 37 °C, 5 % CO₂. At each timepoint the solution was removed from the around the gel block and the gel block was placed on the rheometer plate. Rheology was then carried out using cone and plate. A strain sweep was run from 0.01 to 1 % at 25 °C at a frequency of 1Hz. Frequency sweeps were carried out from 0.01 to 100 Hz at 1 % strain.

2.7 Environmental scanning electron microscopy
Rhodamine tagged magnetoliposomes were made as previously described (Section 2.2). The vesicle-nanoparticle aggregates were resuspended in alginate (1 mL, 2 % wt/vol). The vesicle suspension (0.5 mL) was added to a 24 well plate. Calcium chloride (0.5 mL, 0.1 M) was added to each well and the gel left to cure for 30 minutes. The excess calcium chloride was removed and the gel blocks were washed
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with PBS. A 3T3 cell monolayer at 70 % confluency was separated using Trypsin.EDTA (0.105 mM, 3 mL) for 5 minutes at 37 °C and fresh media (2 mL) added. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant removed. The cell pellet was resuspended in fresh media (5 mL) and a 20 µL sample aliquoted into a haemocytometer to ascertain a cell count. 3T3 cells (35,000) were then seeded on the top of each gel block and fresh media (0.5 mL) was added. The samples were incubated at 37 °C for 14 hours. The media was removed and the samples were washed with PBS (2 mL).

2.8 Patterning alginate vesicle gels
Alginate gel solution was added as a thin layer (~2 mm thick) over a glass slide. Rhodamine vesicle nanoparticle aggregates were added at one end of the alginate solution on the slide and held there magnetically. A small magnet (0.5 mm) was passed from the vesicle suspension to the other end of the slide to magnetically trace a path for the vesicle-nanoparticle assemblies in the gel to follow. The alginate solution was then cured using calcium chloride (0.1 M) and the samples imaged using Nikon Eclipse 50i fluorescence microscope

2.9 Triggered nickel release
Vesicle-nanoparticle alginate solution was added to a trough measuring 25x5x1 mm and covered with a polycarbonate membrane with a 50 nm pore size. Calcium chloride (0.5 mL, 0.1 M) was added over the top of the membrane and the gel was left to cure for 30 minutes. The gel block was cut into sections of 5x5x1 mm and washed with 3 times with PBS (1 mL). Each gel section was added to a cuvette and suspended in calcein blue (2 mL, 0.0156 M). The gel block then received a specific trigger; magnetic pulse or temperature elevation. The fluorescence of the solution in the cuvette was then monitored at 440 nm after excitation at 320 nm. The fluorescence measurement was repeated after 12 hours.

2.10 Nickel release and diffusion through alginate gels of different thicknesses
Vesicle-nanoparticle alginate solution was added at a height of 0.683 mm in troughs measuring 25x5x1 mm, 25x5x2 mm, 25x5x3 mm. The troughs were then filled to the full height using alginate gel solution (2 % wt/vol). The solutions were then covered with polycarbonate membranes with 50 nm pores. Calcium chloride solution (0.5 mL, 0.1 M) was then added and the gel left to cure for 30 minutes. The gel block was then cut into blocks measuring 5x5x1 mm, 5x5x2 mm, 5x5x3 mm and washed using PBS. The gel blocks were added to a cuvette and suspended in calcein blue in PBS (2 mL 0.0156 M) and given a 4 minute magnetic pulse. The fluorescence was then measured at 440 nm every 3 minutes for 30 minutes. The experiment was then repeated after a 15 hour incubation time at room temperature.

2.11 Nickel release with cells
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Vesicle gels were formed from vesicle-nanoparticle aggregates with encapsulated NiCl$_2$ which were magnetically concentrated and suspended in alginate (8 mL) doped with fibronectin (2.27x10$^{-9}$ M) See section 2.7. The gel solution was added to glass vials for cell culture and were seeded with 3T3 cells (20,000 cells/gel) on vesicle gels without magnetic pulse to release the encapsulated nickel, 3T3 cells seeded on alginate with no vesicle-nanoparticle aggregates present and 3T3 cells seeded on alginate with no vesicle-nanoparticle aggregates present with NiCl$_2$ (7.7 mM, 100µL) or (15.4 mM, 100 µL) added to the cell media. Prior to seeding the vesicle gels were added to glass vials (1 mL) and left under UV light for 1 hour. 3T3 cell monolayer at 70 % confluency was separated using Trypsin.EDTA (0.105 mM, 3 mL) for 5 minutes at 37 °C and fresh media (2 mL) added. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant removed. The cell pellet was resuspended in fresh media (5 mL) and a 20 µL sample aliquoted into a haemocytometer to ascertain a cell count. The samples were then seeded 20,000 cells per vial into the gel solution and stirred to ensure mixing. Calcium chloride (0.1 M, 1 mL) was added to each sample to cure the gel. Excess calcium chloride was removed and the gel pellet suspended in fresh media (1 mL). The samples for magnetic release were then placed in an alternating magnetic field for 4 minutes pulsing at 392Hz.

After 4 hours the media was removed from the first samples and the gel blocks resuspended in fresh media (1 mL) and alamar blue (100 µL, 150 µM) was added to each sample and the samples incubated for two hours at 37 °C. The media was removed from the samples and added to a 96 well clear bottom plate in three (200 µL) aliquots. Three repeats were carried out for each sample and fluorescence run at 590 nm after excitation at 530 nm. The gel blocks were resuspended in sterile water (1 mL) and left at -80 °C for 48 hours after which the DNA assay was run. The samples underwent freeze-thawing at -80 °C and 37 °C and were added to a 96 well clear bottom plate in three (50 µL) aliquots with TNE buffer formed from Tris 50 mM, NaCl 140 mM and EDTA 5 mM (50 µL) and Hoescht 33342 stain (100 µL, 1.77x10$^{-5}$ M). Fluorescence was read at 460 nm.

2.12 Imaging magnetically induced cell death
Nickel chloride encapsulated vesicle-nanoparticle aggregates were magnetically purified and resuspended in 0.5 mL of alginate gel solution. The gel solution was seeded with 3T3 cells (20,000 cells/µL). All vesicle nanoparticle aggregates were held at one end of the gel block using NdBF$e$ magnet and the gel block was cured using calcium chloride (0.5 mL, 0.1 M). The excess calcium chloride was removed and the gel blocks washed in PBS (1 mL). The magnet was removed and the gel block suspended in fresh media (1 mL). The samples were incubated for 1 hour at 37 °C, 5% CO$_2$. Live/dead stains were added to sample. Live/ dead stains consist of calcein acetomethoxy derivative which stains live cells green and ethidium homodimer which stains dead cells red. A mixed working solution of calcein acetomethoxy (2 µM) and ethidium homodimer (4 µM) was prepared in PBS and
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aliquots of 500 μL were added to each gel block. The cells were incubated at room temperature for 30 minutes and imaged using a Leica LCS CS5 confocal microscope to scan gel block using 488 nm and 561 nm laser lines. The next samples were pulsed using alternating magnetic field and incubated for 12 hours at 37 °C, 5 % CO2. Live/dead stains were added as previously described and the sample imaged. This was repeated with third gel block after 24 hours.

2.13 Imaging the effect of nickel release on 3T3 cytoskeleton
Vesicle-nanoparticle aggregates which encapsulated nickel chloride were magnetically purified and resuspended in 0.5 mL of alginate gel solution as previously described. The gel was seeded with 100,000 3T3 cells and stirred gently to ensure the cells and vesicles were spread throughout the gel block. The gel was cured using calcium chloride (0.1 M, 0.5 mL), washed with PBS (1 mL) and incubated for 12 hours in fresh media (1 mL) at 37 °C and 5 % CO2. After incubation the gel block was placed in an alternating magnetic field (240s, 352 MHz). The media was then removed and the gel block washed 3 times with PBS. The sample was incubated in paraformaldehyde (1 mL) for 30 minutes at room temperature and then washed with PBS. The cells in the gel block were permeabilised using Triton X-100 (1 % vol/vol in PBS), incubated for 20 minutes at room temperature and then washed six times with PBS. The gel block was blocked using 1 % bovine serum albumin in PBS for 30 minutes at room temperature and washed 5 times with PBS. The gel was incubated with FITC-phallodin (500 μL, 1 % in BSA/PBS solution) for 1 hour, then washed 10 times with PBS and incubated in DAPI in PBS (0.5 mL, 8.5 % vol/vol) for 1 hour at room temperature and finally washed 5 times with PBS. The sample was then mounted and imaged using Leica LCS CS5 confocal microscope.

2.14 Dual release of Ni (II) and calcein
Vesicles were formed from DPPC (12.55 mg, 1.71x10^-5 mol), DMPC (1.27 mg, 1.87x10^-6) and biotin-DHPE (200 μL, 0.2 % mol/mol) were dissolved in chloroform (1 mL) and the solvent removed in vacuo to form a lipid film. Vesicles were also formed from DPPC (14.55 mM 1.9x10^-5 mol) and biotin-DHPE (200 μL, 0.2 mM) were dissolved in chloroform (1 mL) and the solvent removed in vacuo to form a lipid film. Three lipid films were hydrated and extruded as previously described in solutions of 1) Nickel chloride (7.7 mM, 1 mL) in MOPS (pH 7.4) and 2) Calcein (1.6 mM, 1 mL). To give vesicles – DMPC/DPPC with encapsulated nickel chloride, DMPC/DPPC with encapsulated calcein and DPPC with encapsulated nickel chloride. The vesicles were then aggregated with coated magnetic nanoparticles (200 μL, 1.2 mg/mL) with avidin (10 μL, 10 mg/mL) for 1 hour. Vesicle-nanoparticle assemblies were then suspended in alginate solutions containing 1) DMPC/DPPC Calcein and DMPC/DPPC nickel chloride, 2) DMPC/DPPC Calcein and DPPC nickel chloride. The suspensions were added to a trough measuring 25x5x0.5 mm and covered with a polycarbonate membrane with a 50 nm pore size. Calcium
chloride (0.5 mL, 0.1 M) was added over the trough and left to cure for 30 minutes. The resultant gel block was cut into sections of 5x5x0.5 mm and washed three times with MOPS (1 mL, pH 7.4) and suspended in MOPS (4 mL, pH 7.4). The supernatant had aliquots (200 μL) removed every 5 minutes for fluorescence measurement at 520 nm following excitation at 490 nm. The gels were pulsed with an AMF (392 kHz) at 10 minutes for 1 minute and 25 minutes for 4 minutes.

3. Results and Discussion

3.1 Formation of vesicle-magnetic nanoparticle aggregates
Magnetic vesicles were formed by dissolving DPPC and biotin-DHPE in chloroform and removing the solvent in vacuo (Figure 6.2a-d). The lipid film was hydrated in a solution of nickel chloride in PBS. Vesicles were formed by heating to 45 °C, vortexing and extrusion to give uniform 800 nm structures. Coated magnetic nanoparticles were formed as previously described (Chapter 5). Vesicles and coated magnetic nanoparticle solutions, were mixed and aggregated using avidin at room temperature for 60 minutes. These formed large vesicle-nanoparticle structures (Figure 6.2d). The vesicle-nanoparticle aggregates were magnetically purified using a NeBFe magnet. The vesicle-nanoparticle assemblies were resuspended in alginate gel solution (2 % wt/vol). Alginate solution when cured with Ca$^{2+}$ was used to support the vesicle structures and act as a scaffold for cell proliferation (Figure 6.2e).

3.2 Characterisation of vesicle gels
Vesicle gels were studied using a range of techniques including fluorescence microscopy, environmental scanning electron microscopy and rheology. This helped to ascertain the size of the vesicle-nanoparticle aggregates and the location of the vesicle-nanoparticle aggregates throughout the gel block, also the interactions between the vesicles and the interactions between the vesicles and the cells were monitored. These methods were also used to determine the suitability of the vesicle gels as a matrix for cell growth.

In order to visualise the vesicles using fluorescence microscopy, vesicles with 5/6 carboxyfluorescein encapsulated were used. Images showed that large aggregates of vesicles and nanoparticles were formed, which were stable inside the alginate gels both with and without cells present (Figure 6.2e). The vesicle-nanoparticles formed large aggregates, the largest observed was 200 μm. Previous work demonstrated that the aggregates were stable in alginate gels with little spontaneous leakage (Appendix).25

Rheometrical studies indicated that the gels were suitable as a cell scaffold. The elastic modulus was well within documented ranges for used for fibroblast cell growth. Literature data showed that adherent cells grew well in a gel matrix strength between 1-35 KPa.26,27 The properties of the gel changed with incubation and with
cell proliferation. Gel samples which were incubated in PBS did see some increase strength initially than samples incubated in cell media with a higher elastic modulus of $3.5 \times 10^{-3}$ Pa compared to $1.8 \times 10^{-3}$ Pa when gel blocks were incubated in media. This higher elastic modulus in PBS showed that the gels were retaining a higher elasticity. The gel blocks incubated in PBS also showed a longer linear viscoelastic region (not shown), reaching to 100 Hz. In cell media this region only reached to 55 Hz. This shows that the gels incubated in PBS were much more stable.

Rheometric studies showed the gels containing vesicle-nanoparticle assemblies had reproducible properties ($G'$ and $G''$), with viscoelasticity consistent with standard alginate gels, while SEM on other vesicle hydrogels has shown the gel fibrils did not penetrate the vesicle membranes.\(^{28}\)

Rheometrical studies of the gels when cultured as cell scaffolds demonstrated that the presence of cells significantly affected the strength of the gel (Figure 6.3). Gels were incubated without the presence of cells, an elastic modulus of $1.8 \times 10^{-3}$ Pa was observed. This was higher than the elastic modulus of $1.0 \times 10^{-3}$ which was observed when cells were present. Gel blocks were prepared by dispersing cells through a solution of sodium alginate (2 % wt/vol). The gels were then cured by the diffusion of calcium chloride. This created gel blocks with cells dispersed throughout the structure. The presence of cells at gel formation severely weakened the gel structure after several days proliferation, as the presence of the cells disrupted the packing of the gel fibres at gelation which caused a looser interaction creating more fluid like properties in the gel. As has been previously reported, it was known that vesicles interacted with cells when vesicle gels are used as a cell scaffold.\(^{29}\)

### 3.3 Patterning vesicle gels

In order to spatially control and manipulate cellular reactions, controlling the orientation and dispersity of the vesicle-nanoparticle aggregates in the gel blocks was important. Controlling the distribution of vesicle-nanoparticles was done magnetically by using magnets which attracted the nanoparticles present in the aggregates and located the whole vesicle-nanoparticle aggregate to the magnetic source. To visualise this magnetic patterning, vesicles were formed with the membranes doped with rhodamine tagged DHPE (0.1 % in membrane), this gave red vesicles which could be easily visualised using fluorescence microscopy. Thin magnets (500 µm) were then used to trace magnetic paths through the gel solution. The vesicle nanoparticle aggregates were carried along by these magnets giving clearly visible lines of vesicle-nanoparticle aggregates in the alginate solution (Figure 6.4). The gel was cured using calcium chloride fixing the vesicles in place.

Using fluorescence microscopy the lines of vesicle-nanoparticle aggregates were seen as a red line through the gel structure (Figure 6.4a) with a width of ~500 µm. This corresponds to the width of the magnets used to draw the patterns in the gel. In
a closer image of the gel the vesicle-nanoparticle aggregate structures were clearly seen in a 3 dimensional arrangement with large vesicle-nanoparticle aggregates filling areas of the channel following the magnetic pathway (Figure 6.4b).

3.4 Magnetic release
A readily available and bilayer impermeant species was sought for proof-of-principle studies. To this end, nickel (II) salts were ideal to test our methodology. Nickel (II) ions are known to induce cellular cell death at concentrations above 240 μM, and are thought to act through a number of mechanisms. These include the formation of radical oxygen species and the abnormal expression of key genes or enzymes. To determine the concentration of released nickel (II), complexation-induced quenching of the fluorescent dye calcein blue was used (Figure 6.5). The affinity of calcein blue for divalent ions was in the order of $10^8$ M$^{-1}$, so the titration of calcein blue with Ni (II) at the millimolar concentrations expected to be created by magnetic release should give quantitative complexation. Nickel chloride ions quench the fluorescence of calcein blue while calcium ions enhance it under physiological conditions. Calcein blue fluorescence measurements are very sensitive to pH and at high pH values Ca$^{2+}$ ions can also quench the fluorescence of the dye. Titration of a 0.0156 M solution of calcein blue with the nickel (II) released by a 25 μL block of gel containing nickel (II) loaded vesicles (7.7 mM inside the vesicles) revealed that the concentration of Ni (II) inside the gel block was 205 μM (4.7 % encapsulation efficiency), but also revealed that the diffusion of this cation out of the anionic gel matrix of the gel block was significantly retarded compared to 5/6 CF, only becoming complete after 5 hours (Figure 6.6a).

The rate at which the Ni (II) cation diffused through an alginate gel matrix was estimated by encasing nickel-loaded vesicle-nanoparticle assemblies within different thicknesses of calcium alginate gel. Although low levels of release occurred from the exposed sides of the gel block, each additional millimetre of gel significantly slowed the release of the nickel ions (Figure 6.6b). The released nickel ion concentration had equilibrated after 15 h (data not shown). The retardation of Ni (II) diffusion through calcium alginate suggested the cation interacted with the anionic alginate polysaccharide, either transiently or in an exchange reaction with the calcium counterions.

Fluorescence microscopy revealed that the cells and magnetic nanoparticle-vesicle assemblies in the gel were in close proximity, an observation that suggested the biotin-coated vesicle-nanoparticle aggregates and fibroblasts weakly interacted. Therefore despite the relatively slow response of most cell types to environmental Ni (II) (cell death usually occurs over a short time period of hours to days), exposure to Ni (II) should occur relatively quickly after the AMF, followed by a cellular response within 24 h.
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3.5 Magnetically induced cell death

The cell type chosen for these studies were 3T3 fibroblasts, a robust cell line known to proliferate on a variety of synthetic biomaterials. Nonetheless, fibroblasts were known to interact poorly with pure calcium alginate hydrogels due to a lack of cellular attachment points.

In order to overcome this limitation calcium alginate hydrogels were doped with fibronectin, a high molecular weight glycoprotein found in the extracellular matrix, which significantly enhanced cell adhesion and has been known to play a role in cell differentiation and migration. Indeed we found that adding fibronectin \((2.27 \times 10^{-9} \text{ M})\) dramatically increased fibroblast viability but without compromising the integrity of the Ni (II)-containing vesicles. Fibronectin-doped \((2.27 \times 10^{-9} \text{ M})\) alginate gels containing fibroblasts seeded at a density of 20,000 per 500 mm\(^3\) (0.5 mL) were fabricated in glass vials (2.3 cm diameter) as detailed previously. Control gels were also fabricated that contained coated magnetic nanoparticles only, Ni (II)-containing vesicles at a concentration of 15.4 mM or 7.7 mM only and no external stimulation, and with external Ni (II) added to the sample (200 µL, 15.4 mM). Media was added to cover the gel (1 mL) and the sample incubated at 37 °C. Analysis of the cellular response was carried out at regular intervals over 24 h, with cell number analysed by the DNA assay (using Hoechst 33258) and metabolic activity analysed using the Alamar blue assay. The data was obtained by comparison with data in a standard curve of the fluorescence with a known number of cells.

In the absence of either of the active components, Ni (II)-containing vesicles and coated magnetic nanoparticles, exposure to a 392 kHz AMF did not affect the proliferation of the fibroblasts in the fibronectin-doped alginate matrix, with an increase in cell number (Figure 6.7). However application of the AMF to the Ni (II)-containing magnetic vesicle gels caused a dramatic decrease in the viability of the cells, with strong decreases in cell number (Figure 6.7) and after 24 hours, >95 % of the cells in the hydrogel had died. This decrease in cell viability mirrored that observed if nickel (II) chloride (200 µl, 15.4 mM) was added to the surrounding solution, suggesting that release of the toxic Ni (II) ions has induced death in the cultured cells.

3.6 Time controlled cell death

The slow diffusion of the nickel ions through the gel also allowed the propagation of Ni (II)-induced cell death to be observed directly. The alginate gel can be patterned by placing a strong NdFeB permanent magnet on the side of the cell culture chamber, which pulls the Ni (II)-vesicle-coated nanoparticle assemblies to one side and holds them in place while the gel is formed by calcium (II) infusion. The patterned gel was subjected to the AMF, releasing the Ni (II) ions, which then diffused slowly through the gel from the side of the chamber. A live-dead assay
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across the gel block revealed that cell death progressively extended from the Ni (II) containing region, with those cells closest to the Ni (II) releasing region dying within 12 hours, followed by cell death throughout the material after 24 hours (Figure 6.8). Further imaging studies of the magnetically induced cell death showed both the cytotoxicity of nickel and the degradation of the cell as the apoptotic process progressed. The different apoptotic pathways triggered by the nickel have been extensively studied. The two main triggers of cell death appeared to be centred on the mitochondria and DNA. In order to observe the apoptotic process, cells which had undergone magnetically induced cell death were studied using immunofluorescence (Figure 6.9). These studies showed that the cytoskeleton appears to degrade as the cells undergo cell death (Figure 6.9 e,f). The nuclei are also seen to be misshapen in the last stages of cell death, suggesting that the DNA is being destroyed (Figure 6.9 g,h). The nuclei remained intact as no DAPI staining was observed in the cytoplasm of the cell.

3.7 Dual release
The complexation between nickel and calcein also made it possible to study the thermal release transition exhibited by the vesicles and the behaviour of the released nickel. When the vesicle membrane changed between liquid crystalline phases it exhibited a characteristic transition temperature. This transition was responsible for the change in membrane permeability which caused the release from the vesicle core. Vesicles formed DPPC have a transition temperature of 42 °C, while vesicles formed from a combination of DPPC (87.5 %) and DMPC (12.5 %) have a lower transition temperature of 37 °C. This temperature difference allowed the delivery of different compounds on the receipt of different triggers. Vesicles with calcein encapsulated were formed from DMPC:DPPC mixture, whereas vesicles encapsulating nickel were formed from DMPC:DPPC mixture and from DPPC. All vesicles were doped with biotin-DHPE (0.2 % mol/mol) and aggregated with coated magnetic nanoparticles. As can be seen in Figure 6.10, when vesicles containing nickel chloride and vesicles containing calcein both formed from the DMPC:DPPC were held together and an AMF pulse was used to trigger release little change in fluorescence was seen.

This was because the nickel and the calcein were both released simultaneously and the calcein fluorescence was immediately quenched. However when the calcein vesicles were formed from the DMPC:DPPC mixture and the nickel vesicles were DPPC only, clear changes in the fluorescence were observed on the receipt of different triggers. A one minute alternating magnetic field pulse was enough to trigger release of calcein from the DMPC:DPPC mixture while the nickel remained encapsulated. A further 3 minute pulse then released the nickel from the DPPC vesicles quenching the fluorescence of the released calcein. This showed that the vesicles of altered lipid mixtures can be designed to respond separately on the receipt
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of different triggers, releasing several active molecules individually to interact with cells in the surrounding environment.

4. Conclusion
This study has shown vesicle gels can be easily formed and patterned magnetically. Although the mechanical properties of the gels change during cell culture, the vesicle gels were not cytotoxic and supported cell growth. When nickel chloride was encapsulated into the vesicles, controlled release was obtained to give cell death. This occurred even though nickel (II) ions interacted strongly with the alginate gel matrix. Nickel chloride caused cell death of the cell samples at encapsulated concentrations of 15.4 mM. This induced cell death was controlled non-invasively using a remote alternating magnetic field. In future studies we will investigate targeting specific cell lines through possible vesicle-cell interactions such as antibody labelling of the vesicle membrane and exploring the possibility of targeted differentiation of stem cells.

Figures

Figure 6.1 Magnetic release of encapsulated material from vesicle gels.
Figure 6.2 a-c) Schematic to show the steps of forming vesicle alginate gel with vesicle nanoparticle aggregates and the encapsulation of materials. d) Fluorescence microscopy of vesicle-nanoparticle aggregates with 5/6-carboxyfluorescein encapsulated in the alginate gel block. e) Vesicle-nanoparticle aggregates in an alginate gel with 3T3 cells stained with FITC-phalloidin and DAPI. f) Vesicle-nanoparticle aggregates located on the cell surface in a cross section of the gel by environmental SEM all scale bars are 25 µm.
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Figure 6.3 Rheological studies of vesicle gels of the elastic modulus over time gels incubated in PBS (white), gels incubated in media supplemented with fetal bovine serum (black) and gels incubated in media and seeded with 3T3 cells (grey) n=3.

Figure 6.4 A) Magnetically positioned vesicle-nanoparticle aggregates in alginate gels scale bar 500 µm. B) Higher magnification of image A, scale bar 100 µm.

Figure 6.5 Calcein blue chelating a Ni (II) ion.
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Figure 6.6 Magnetic release of nickel chloride from vesicle gels. a) shows the relative release rates of 5/6-carboxyfluorescein (black) and nickel chloride (white). b) shows the release of nickel chloride from different thickness alginate blocks as the nickel quenches the calcein blue solution after 30 minutes (black) and after 15 hours (white).
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Figure 6.7 b) Nickel chloride encapsulating vesicle gels interacting with 3T3 cells. 3T3 cells cultured on alginate with no vesicle-nanoparticle assemblies present (square), with vesicle-nanoparticle aggregates present but with no magnetic release (diamond), vesicles encapsulating nickel chloride with magnetic release (circle) and no vesicles present and free nickel chloride (15.4 mM, 200 µl) (triangle).

Figure 6.8 Confocal microscopy tile scans showing the diffusion of nickel chloride through the gel triggering apoptosis, showing the 3T3 cells uptake of calcein and ethidium homodimer in the live/dead assay with a) before magnet pulse, b) after 12 hours and c) after 24 hours.
Figure 6.9 Confocal micrographs a-d) showing the deterioration of the cell population over time. The f-actin of the cell is labelled with FITC phalloidin and the nuclei with DAPI. a) 6 hours after the addition of nickel, b) 12 hours, c) 18 hours and d) 24 hours. e-f) show the degradation of the cytoskeleton of the cell labelled green with FITC tagged phalloidin after 12 hours. g-h) show the misshapen nuclei from the apoptotic process labelled blue with DAPI after 18 hours all scale bars are 25 µm.
**Figure 6.10** Release of nickel chloride and calcein from vesicles. Gel samples had DMPC:DPPC vesicles encapsulating nickel chloride and DMPC: DPPC vesicles encapsulating calcein (white). Samples had DMPC :DPPC vesicles encapsulating calcein and DPPC vesicles encapsulating nickel chloride (black). A 1 minute alternating magnetic field pulse was used at 10 minutes and a 3 minute alternating magnetic field pulse was used at 25 minutes. Data was normalised to maximum release.
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Chapter 6  
Magnetically Triggered Differentiation in Mouse Embryonic Stem Cells  
F de Cogan, C Merry, SJ Webb, JE Gough

This chapter contains preliminary data carried out to trial the vesicle-nanoparticle system as a possible tool for embryonic stem cell differentiation
Chapter 6 Magnetically Induced Differentiation in Mouse Embryonic Stem Cells

Magnetically Triggered Differentiation in Mouse Embryonic Stem Cells

Felicity de Cogan, Catherine Merry, Simon Webb and Julie Gough

Abstract
Forming biomaterials that replicate the complex structure and functions of tissue has so far proved to be challenging. The possible applications of such mimics are wide ranging from subcutaneous drug delivery and tissue engineering, to differentiation of pluripotent stem cell populations. Vesicles can act as active stores with drugs encapsulated, ready for controlled release. This work explores the interactions of embryonic stem cells with large alginate hydrogel matrix used to support vesicle-nanoparticle assemblies and the use of vesicle-nanoparticle aggregates as possible initiators of stem cell differentiation. The vesicle-nanoparticle assemblies encapsulate molecules known to trigger differentiation in E Cadherin\textsuperscript{(-/-)} cells, such as SB431542 (4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]-benzamide) and release it magnetically on the receipt of a trigger causing differentiation to occur.

1. Introduction
Embryonic stem cells (ES cells) are pluripotent cells, derived from embryos at the blastocyst stage of embryogenesis.\textsuperscript{303} The pluripotency of ES cells gives them the ability to differentiate into the three germ layers: endoderm, ectoderm and mesoderm.\textsuperscript{1} Stem cells have the potential to revolutionise medicine through the elucidation of disease process and the possible uses of stem cells in regenerative medicine. Stem cells are commonly grown in a two dimensional environment \textit{in vitro} on gelatin coated tissue culture plastic.\textsuperscript{312} Culture conditions are vital for maintaining stem cell pluripotency. The addition of growth factors such as leukemia inhibitory factor (LIF) were needed to allow stem cells to be cultured on matrices in the absence of feeder cells.\textsuperscript{311} Genetically modified stem cells are very useful for investigations into the interactions of stem cells with each other and the immediate environment surrounding them. One stem cells strain which exemplifies this is the E Cadherin\textsuperscript{(-/-)} line. E Cadherin is from the classical Cadherin family.\textsuperscript{2} It is expressed on embryonic stem cells and most epithelial cells.\textsuperscript{3} E Cadherin mediates cell-cell contact.\textsuperscript{4} It is composed of a cytoplasmic region, a lipophilic region which is embedded in the cell membrane and an extracellular component.\textsuperscript{5} The external section interacts with E Cadherin proteins on neighbouring cells in a calcium dependent manner.\textsuperscript{6} E Cadherin interacts with the actin skeleton of the cell by binding beta catenin in the cytoplasmic region.\textsuperscript{7} Beta catenin communicates with the contents of the cells cytoplasm via alpha catenin.\textsuperscript{8} p120 catenin is also used to stabilise E Cadherin by preventing the endocytosis of the protein from the membrane.\textsuperscript{9} E Cadherin is vital for embryo
development and embryos which do not express it fail at the blastocyst stage.\textsuperscript{10} E Cadherin is also associated with tumour cell metastasis.\textsuperscript{11} When E Cadherin is removed from the stem cell population, the cells no longer form ES cell colonies, but remain pluripotent.

There have been many different studies carried out culturing stem cells on different matrices including hydrogels such as alginate.\textsuperscript{12} Although stem cells can maintain pluripotency, this often occurs as the stem cells form embryoid bodies creating micro environments in the alginate which support their needs. Alginate has often been used as a differentiation matrix for stem cells, particularly to derive mesoderm cells.\textsuperscript{13} Stem cells which have been cultured in alginate beads often form embryoid bodies which were easy to extract from the matrix.\textsuperscript{14}

To achieve differentiation of pluripotent stem cells, there are several possible pathways. Cells have been grown in differentiation media, such as N2B27 which provided the requirements for neural cells (not pluripotent cells).\textsuperscript{15} This gave rise to general differentiation with a majority of cells following a specific differentiation path.\textsuperscript{16} Single compounds such as retinoic acid have been added to the stem cell media which triggered cell differentiation.\textsuperscript{17,18} Changes to the cell environment by growing cells on different scaffolds have also triggered differentiation.\textsuperscript{19} All of these methods used changes which affected the whole cell population. These processes were also dependent on invasive changes which can be difficult to carry out \textit{in vivo} or in a complex three dimensional \textit{in vitro} environment. However, based on previous work, our studies showed that it was possible to encapsulate and release biologically active compounds non-invasively using an AMF.

Vesicles are commonly used as drug delivery agents as they can encapsulate both hydrophobic and hydrophilic compounds.\textsuperscript{20} Hydrophobic compounds are encapsulated at the lipid mixture stage and are embedded in the hydrophobic region of the phospholipid bilayer. Hydrophilic compounds are encapsulated in the vesicle formation stage and are held in the aqueous core. Phospholipid vesicles have generated a lot of interest for drug delivery as they can be used selectively to release encapsulated contents on the receipt of an external trigger.\textsuperscript{68} Magnetic nanoparticles also have extensive medicinal uses ranging from imaging,\textsuperscript{21} drug delivery\textsuperscript{22} and tumour targeted hyperthermia.\textsuperscript{23} Nanoparticles are formed by coprecipitation of Fe (II) and Fe (III) in a basic environment in the absence of oxygen to give Fe$_3$O$_4$ as single domain magnetic nanoparticles.\textsuperscript{103} The surface of the nanoparticles is easily modified through iron chelating groups to create an interactive layer, which also allows the nanoparticles to be dispersed in both hydrophobic and hydrophilic environments.\textsuperscript{24} One of the most utilised properties of the magnetic nanoparticles is the ability to generate localised heat.\textsuperscript{86} When the nanoparticles are placed in an alternating magnetic field the nanoparticles continually align with the changing field. This motion generates energy, which is then released as thermal energy through Ne\textaelrelaxation and Brownian motion.\textsuperscript{86}
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Phospholipid vesicles and magnetic nanoparticles have been often combined to create ‘Magnetoliposomes’. These have unique properties as they can be used as active chemical stores that can be controlled using an external magnet. Most literature focuses either on encapsulating the nanoparticles in the aqueous core of the vesicles or embedding the nanoparticles in the hydrophobic bilayer. However Webb et al reported a method for building vesicle-nanoparticle structures where nanoparticles were closely linked but held externally to the vesicles. These vesicle-nanoparticle aggregates have previously been shown to be very successful for delivering biomolecules.

Phospholipid vesicle-nanoparticle assemblies are more stable when supported in a three dimensional matrix. The most suitable matrix to use is a hydrogel to maintain the correct environment and biocompatibility. The gel also needs to be formed using a method which does not cause vesicle damage such as changes in temperature, pH and sonication. The gel matrix must also be carefully screened so that it does not penetrate the vesicles as it is formed. Alginate gels formed from sodium alginate and cured using calcium chloride, have been shown to support DPPC vesicles well. These alginate gels show no spontaneous leakage and are biocompatible. Alginate is isolated from brown algae and is not recognised by mammalian cells and it should trigger a low immune response.

Herein we report using the vesicle-nanoparticle assemblies in hydrogels as a possible scaffold for stem cell growth. These ‘magnetic vesicle gels’ act as an interactive cell scaffold, which can be used to trigger differentiation after a magnetic signal. We will use vesicles to encapsulate small molecule differentiation triggers, which can be released using an alternating magnetic field to differentiate both E14 a robust cell line derived from mouse neural stem cells and E Cadherin(-/-) cells, which have been genetically modified to remove the E Cadherin gene. We also study extensively the interaction of embryonic stem cells with alginate, particularly for propagation, maintaining pluripotency and extraction from the gel matrix.

2. Materials and Methods

2.1 E14 stem cell culture

Cells were cultured on gelatin coated tissue culture plastic. Plates and flasks were gelatinised by the addition of 0.1 % gelatin in H2O to each well or flask, followed by incubation at room temperature for 30 minutes. Prior to cell seeding, excess gelatin was removed by pipette from the tissue culture plastic.

Cells were cultured in Knockout DMEM media supplemented with fetal calf serum (10 %, vol/vol), L-glutamine (1 % vol/vol), MEM non-essential amino acids (1% vol/vol), 2-mercaptoethanol (0.01 %, vol/vol) and LIF 1000 U/mL.

To passage cells, the cell media was removed and the cells washed with PBS. Trypsin/EDTA was added (0.105 mM, 3 mL) to the cells. The cells were incubated
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for 3 minutes at room temperature and the trypsin was then neutralised with cell media (2 mL). The cells were typically split at either 1:6 or 1:10 ratios onto new gelatinised culture plates.

Cells were routinely frozen. After trypsinisation the cells were added to a 10 mL falcon tube and centrifuged at 700 rpm for 3 minutes. The media was removed and the cell pellet was resuspended in new media supplemented with DMSO (10 % vol/vol). The cell suspension was transferred to cryovials and slowly frozen to -80 °C. The vials were then transferred to liquid nitrogen.

2.2 Two dimensional culture of E14 cells on gel scaffolds; alginate, alginate doped with fibronectin and gelatin.

Gelatin coated 6 well plates were prepared. Alginate (2 % wt/vol, 1 mL) was added to well plates and cured by the addition of calcium chloride (1 mL, 100 mM). The excess solution was removed and the gels washed with PBS. Alginate doped with fibronectin was prepared by the addition of fibronectin from bovine plasma (2.27x10^{-9} M) into the alginate solution prior to addition to the well plate (2 % wt/vol, 1 mL). The gel was cured using calcium chloride (100 mM, 1 mL). Any excess solution was removed and the gel washed with PBS. Cells were harvested from confluent flasks and seeded onto gel surfaces and the gelatinised well plate at a density of 100,000 cells/mL. The gels were suspended in fresh media (1 mL) and incubated at 37 °C and CO₂ (5 % vol/vol).

2.3 Three dimensional culture of E14 cells on gel scaffolds; alginate, alginate doped with fibronectin and gelatin.

Gelatin coated 6 well plates were prepared. Alginate (2 % wt/vol, 1 mL) was added to each well of a plate and cells at a density of 100,000 cells/mL were added into the alginate. The solution was stirred gently to ensure mixing and the gel was cured by the addition of calcium chloride (1 mL, 100 mM). The excess solution was removed and the gels washed with PBS. Alginate doped with fibronectin was prepared by the addition of fibronectin from bovine plasma (2.27x10^{-9} M) into the alginate solution prior to addition to the well plate (2 % wt/vol, 1 mL) to each well. Cells were prepared at a density of 100,000 cells/mL and were added into the alginate gels. The solution was stirred gently to ensure mixing and the gel was cured by the addition of calcium chloride (1 mL, 100 mM). The excess solution was removed and the gels washed with PBS. Gelatin (1 mL) was added to each well of the plate and the cells were seeded into the gelatin solution at a density of 100,000 cells/mL. All the gels were suspended in fresh media (1 mL) and incubated at 37 °C and CO₂ (5 % vol/vol).
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2.4 EDTA mediated gel disruption.
The gel samples containing cells had the media removed and were washed with PBS. The gels were then incubated in EDTA in PBS (2 mL) for 30 minutes at 37 °C at varying concentrations (1 mM, 5 mM, 10 mM, 50 mM, 100 mM). The solutions were centrifuged at 1000 rpm for 5 minutes and the solution was removed from the pellet. The pellet was resuspended in cold PBS (1 mL) and a cell count taken.

2.5 Calcium dependence of alginate gel formation.
Alginate gel solutions containing E14 cells were formed as previously described (Section 2.3). The cell alginate suspensions were added to 6 well plates and were incubated with calcium chloride solutions (1 mL) with a varying range of concentrations (1 mM, 5 mM, 10 mM, 50 mM, 100 mM) and incubated for 30 minutes. The excess solution was removed and the gel blocks were suspended in fresh cell media (1 mL).

2.6 Ascorbic acid mediated differentiation.
On a gelatinised 6 well plate freshly harvested E14 cells were seeded at a density of 100,000 cells/well. The cells were incubated in cell media (2 mL) supplemented with ascorbic acid-2-phosphate (0.25 mL, 4.3 mM). The samples were then incubated at 37 °C and CO₂ (5 % vol/vol).

2.7 Neural differentiation
The cell media used was N2B27. N2B27 media consists of 1:1 ratio of neurobasal media and DMEM F12 supplemented with 50 μg/mL BSA (fraction V), 500 μM L-glutamine, 5 mL N2 and 10 mL B27 supplements.

A confluent flask of E14 cells was harvested and the cell suspension centrifuged at 700 rpm for 3 minutes. The media was removed and the cell pellet was resuspended in fresh N2B27 media (12 mL) and split at 1:6 ratio into a freshly gelatinised 6 well culture plate (2 mL) per well. The samples were then incubated at 37 °C and CO₂ (5 % vol/vol). Fresh media was added every 48 hours. After 14 days the media was removed and the cells washed with cold PBS (2 mL). Paraformaldehyde (4 % vol/vol, 2 mL) was added to each well and the plate incubated in the fridge for 10 minutes. The paraformaldehyde was removed and the samples washed twice with PBS (2 mL). PBS (3.5 mL) was then added to each plate and they were stored overnight at 4 °C. The PBS was removed and the cells incubated with block solution (block solution contains goats serum 1 % vol/vol, bovine serum albumin 0.1 %, 1mg/mL and Triton X-100 0.1 % vol/vol in PBS) for 30 minutes. The block solution was removed and each well had primary antibodies β III tubulin (monoclonal mouse IgG) 1:200 dilution in block solution and Oct4 (rabbit polyclonal IgG) 1:200 dilution in block solution added. The samples were incubated at room temperature for 60 minutes. Each well was washed with PBS (2 mL) five times. The secondary
antibodies were then added, Alexa Fluor-488 goat anti-mouse IgG diluted in block solution (1:1000) and Alexa Fluor-546 (goat anti-rabbit IgG) diluted in block solution (1:1000) respectively. The samples were incubated at room temperature for 60 minutes in the dark. The solutions were removed and each well washed with PBS five times (2 mL). The cell nuclei were labelled by incubating with DAPI (10 µl, 3 nM) in PBS at room temperature in the dark and mounted using Prolong Gold antifade (Molecular probes P36934).

2.8 E-Cad<sup>+/−</sup> cell culture

Cells were cultured on gelatin coated tissue culture plastic. Plates and flasks were gelatinised by the addition of 0.1% gelatin in H<sub>2</sub>O to each well or flask incubating at room temperature for 30 minutes. Prior to cell seeding, excess gelatin was removed from the tissue culture plastic using a pipette.

Cells were cultured in Knockout DMEM media supplemented with fetal calf serum (10 %, vol/vol), L-glutamine (1 % vol/vol), MEM non-essential amino acids (1% vol/vol), 2-mercaptoethanol (0.01 %, vol/vol) and LIF 1000 U/mL.

To passage cells the cell media was removed and the cells washed with PBS. Trypsin/EDTA (0.105 mM, 3 mL) was added to the cells, and the samples were incubated for 3 minutes at room temperature. The trypsin was then neutralised with cell media (2 mL) respectively. The cells were typically split at either 1:6 or 1:10 ratios onto new gelatinised culture plates.

Cells were routinely frozen. After trypsinisation the cells were added to a 10 mL falcon tube and centrifuged at 700 rpm for 3 minutes. The media was removed and the cell pellet was resuspended in new media supplemented with DMSO (10 % vol/vol). The cell suspension was transferred to cryovials and slowly frozen to -80 °C. The vials were then transferred to liquid nitrogen.

2.9 E-Cad<sup>+/−</sup> cell culture in media without LIF

Cells were cultured on gelatin coated tissue culture plastic. Plates and flasks were gelatinised by the addition of 0.1% gelatin in H<sub>2</sub>O to each well or flask incubating at room temperature for 30 minutes. Prior to cell seeding, excess gelatin was removed from the tissue culture plastic using a pipette.

Cells were cultured in Knockout DMEM media supplemented with fetal calf serum (10 %, vol/vol), L-glutamine (1 % vol/vol), MEM non-essential amino acids (1% vol/vol), 2-mercaptoethanol (0.01 %, vol/vol) and LIF 1000 U/mL. Every 48 hours the cells were split 1:6 and the concentration of LIF in the media was decreased by 25 % until no LIF was present.
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2.10 Encapsulation of SB431542 inside vesicles

DPPC, (14.55 mg, 1.9x10^{-5} mol) and N-(biotinoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Biotin-DHPE), (0.2 % mol/mol) were dissolved in chloroform (1 mL). The solvent was then removed in vacuo. The lipid film was rehydrated in SB 431542 (1 mL, 0.957 mM) in PBS, heated to 50 °C and vortexed. The lipid mixture was extruded 19 times through a single ~800 nm pore membrane.

2.11 Release of SB431542 from vesicle gels

The vesicles were purified using an equilibrated PD-10 sephadex column. The sephadex column was equilibrated by the addition of PBS (25 mL). The vesicle suspension in PBS was added to the column (2.5 mL), the vesicles were then eluted using PBS (3 mL). The vesicles were then aggregated with magnetic nanoparticles synthesised as previously described (Chapter 6) (200 µL, 1.2 mg/mL) and avidin (10 µL, 10 mg/mL) for 12 hours then magnetically purified using NdBFe magnet. The vesicles were resuspended in alginate gel solution (1 mL, 2 % wt/vol) and placed into 25x5x5 mm trough. The gel was incubated under calcium chloride (0.1 M, 500 µL) for 30 minutes at room temperature and cut to 5x5x5 mm blocks. The blocks were suspended in 2 ml PBS and pulsed using an alternating magnetic field (392 Hz, 4 minutes) the solution was then aliquoted at 30 minute intervals and the fluorescence measured at 485 nm after excitation at 420 nm.

2.12 Vesicle gels encapsulating SB431542 as cell scaffolds.

E Cadherin^{+/-} cells were seeded at a density of 100,000 cells/mL and cultured on gelatin coated tissue culture plastic (as described above) in media (2 mL) with no LIF present. Cell samples were cultured with i) with nothing added to the culture, ii) with 0.768 µL of DMSO added to the culture well, iii) with 0.768 µl of SB431542 (0.026 M) in DMSO added to the culture well, iv) with 200 µL of vesicle-nanoparticle aggregates with SB431542 encapsulated (0.957 mM), v) with 200 µL of vesicle-nanoparticle aggregates with SB431542 encapsulated (0.957 mM) which have been exposed to an AMF pulse (392 Hz, 4 minutes), vi) 200 µL of vesicle-nanoparticle aggregates with PBS encapsulated. The samples were incubated for 5 days and the media was changed every 48 hours. The samples were imaged using Nikon Eclipse 50i. fluorescent microscope. The media was then removed from the samples and they were washed with PBS (2 mL) and paraformaldehyde (4 % vol/vol, 2 mL) was added to each well and the plate incubated in the fridge for 10 minutes. The paraformaldehyde was removed and the samples washed twice with PBS (2 mL). PBS (3.5 mL) was then added to each plate and they were stored overnight at 4 °C. The PBS was removed and the cells incubated with block solution (block solution (described in section 2.7) for 30 minutes. The block solution was removed and the samples were incubated with primary antibody Rabbit anti nanog polyclonal
antibody in a 1:500 dilution in block solution for 60 minutes. The cells were washed with PBS five times and incubated with secondary antibody Alexa Fluor 488 goat anti rabbit IgG in a 1:1000 dilution for 60 minutes at room temperature in the dark. The cells were then washed with PBS five times and incubated with DAPI (3 µM in PBS) at room temperature in the dark and mounted using Prolong Gold antifade.

3 Results and Discussion

3.1 Stem cell growth on alginate gels.

Previously phospholipid vesicle-nanoparticle assemblies had used alginate as a supporting 3D matrix, so we tried to encourage stem cell growth in the alginate matrix while maintaining pluripotency. Most literature showed that alginate is used as a scaffold to assist stem cell differentiation, particularly chondrogenic differentiation. However, alginate has been used successfully as a cell scaffold for a range of primary and stem cells. Stem cells were typically encapsulated in small alginate beads creating microenvironments which were easily controlled. The process of encapsulating vesicle-nanoparticle assemblies and stem cells into microbeads was extremely complex and so large alginate gel blocks of ~1.5 cm diameter were investigated as cell scaffolds. Alginate gel blocks have previously been demonstrated to encapsulate vesicle-nanoparticle assemblies well, with little spontaneous leakage and no vesicle damage during the gelation process. To examine the interaction of stem cells with alginate, E14 cells were grown on the surface of alginate blocks. Alginate doped with fibronectin (2.27x10^{-9} M) was also used as this had been shown to increase attachment in fibroblast cell lines. The results showed that after 72 hours E14 stem cells had survived on the surface of both the alginate and alginate doped with fibronectin (Figure 7.1). Small colonies were also formed. However, in comparison to control samples on gelatin coated tissue culture plastic which is standard culture conditions for E14 cells, the cell proliferation was low. Quantitative assays reflected the microscopy images, where low levels of cell proliferation was seen and small colonies identified (Figure 7.1). The cells were removed from the gel blocks using trypsin, centrifuged and replaced in fresh media. The cells were then counted (Figure 7.1). These data showed that, despite the seemingly sparse population, the population doubled over the 72 hour period. Nonetheless, this was considerably lower than the control, which showed a population doubling in the first 12 hours. It was promising that the trypan blue assay showed that the cells removed from the surface of the alginate were live, viable cells (Chapter 2, Section 2.10.1). There appeared to be no difference in cell proliferation between the alginate and the alginate doped with fibronectin gels. When cells were detached from the gel surface they were replated onto gelatin coated TCP and pluripotency was assessed but very few cells attached and little proliferation was seen. As E14 cells did not recognise alginate (as it is not mammalian) it was possible that cells lost some attachment ability after prolonged culture.
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3.2 Encapsulation of stem cells in alginate gel blocks.
In order for the vesicle-nanoparticle aggregates to interact well with the stem cell population, the cells need to be in close proximity to the vesicle-nanoparticle assemblies, inside the gel block. Seeding the cells into the gel block instead of on the surface also mirrors the three dimensional in vivo environment more closely. Freshly trypsinised E14 cells were suspended in either sodium alginate or sodium alginate doped with fibronectin (2.27x10^{-9} M) and added to a culture plate. The gels were then cured on the addition of calcium chloride, trapping the cells inside the gel matrix. However, microscopy on the gel blocks revealed that this was not an ideal environment for the cells (Figure 7.1). The cell population was sparse throughout the gel and the cells which remained had formed embryoid bodies. Embryoid body formation was not desirable as differentiation often occurs in the inner cells due to the crowded environment. Embryoid body formation can also contribute to a decrease in cell number as cells in the core of the body die due to lack of oxygen and nutrients. Control samples were cells were cultured in a pseudo three dimensional gelatin gel, which did not give a true three dimensional environment as the thermoresponsiveness of gelatin meant that the gel was fluid at room temperature. The excess of gelatin did not appear to hinder cell proliferation or colony formation at 72 hours.

Quantitative studies into the encapsulation of the E14 cells in the gel block supported the images in (Figure 7.1). Cells counted using trypan blue assay showed that not all the cells encapsulated in the gel were live and viable (Figure 7.1). The cells did not grow in either of the alginate gels, showing again that alginate doped with fibronectin cannot be used to enhance cell attachment. Cell proliferation in the alginate gels began to decrease towards 72 hours, as the cells which had survived encapsulation and formed embryoid bodies died due to the lack of oxygen and nutrient diffusion through the surrounding gel and cell mass. Cell proliferation in the gelatin control was also slower initially, as the excess gelatin slowed the diffusion of oxygen and nutrients to the cells. However, when the cell media was changed at 24 hours, excess gelatin in its liquid form was removed. After this the standard proliferation pattern was observed. It was not possible to remove the cells from inside the alginate gel matrix and the alginate gel matrix doped with fibronectin to test pluripotency using standard methods.

3.3 Extraction of cells from alginate using EDTA
In order to try to remove the cells encapsulated inside the 3D gel block, the gel block was dissolved using ethylenediaminetetraacetic acid (EDTA). EDTA chelates to the calcium ions which hold the strands of alginate together to form a matrix. When the calcium is removed, the gel turns back into a liquid. EDTA (0.1 M) was added to the gel blocks and the cells were removed from the gel debris by centrifugation. The
cells were then replated on gelatin coated tissue culture plastic to assess pluripotency (Figure 7.2). Preliminary data in Figure 7.2 showed that only a few cells survived. Although EDTA has been used to recover cells from the gel matrix, it appeared to have a detrimental effect on the ES cells. Cells incubated from alginate gel, alginate doped with fibronectin and gelatin gel blocks all showed this effect, therefore it was not solely due to interactions with the alginate gel but is due to the EDTA. We suggest that cells cannot survive incubation in EDTA at high concentrations. In order to find a process to extract the cells from the gel blocks several methods were trialled.

As cell dissociation buffer detaches cells by interfering with calcium dependent attachment points, we hypothesised that it may be possible to use cell dissociation buffer to remove calcium ions from the gel matrix. When alginate gel blocks were incubated in cell dissociation buffer for 30 minutes, 1 hour and 4 hours, the gel remained intact and the cells could not be removed. The cell dissociation buffer was used to detach cells from gelatin coated TCP in 30 minutes. When these cells were replated on gelatin coated TCP, they adhered and proliferated. Trypan Blue assay showed that the cell dissociation buffer did not destroy the gel matrix and it did not harm the cells.

Sodium citrate has previously been shown to degrade alginate beads. This occurs by removing the calcium ions from the gel through complexation with the citrate. Sodium citrate was buffered using MOPs and gel blocks were incubated for 30 minutes. The alginate blocks remained intact and the cells could not be retrieved. The cells cultured on gelatin were not removed by sodium citrate and were washed, trypsinised and replated on gelatin coated TCP. The sodium citrate appeared to have little effect on the cells as the cells adhered and proliferated.

3.4 Calcium dependent alginate formation

As EDTA was the only compound which successfully disrupted the alginate matrix to allow cell removal, the effect of EDTA concentration on the gels and cells was studied in order to find an optimum concentration which would disrupt the gel network but not destroy the cells (Figure 7.2). All EDTA solutions of 10 mM or less showed no matrix disruption. When incubated in 50 mM of EDTA, some matrix disruption was observed but not enough to remove the cells from the matrix. When the gel block was subjected to severe agitation, the cells could be removed but did not survive the extraction process. At concentrations higher than 50 mM the gel network was disrupted but the cells did not survive, implying that EDTA could not be used to isolate the cells. To try and create an alginate gel which could be used for cell culture, and from which the cells could be isolated, investigations into the dependence of the gel on the calcium ion concentrations were carried out. Alginate
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gels were cured using calcium chloride ions at a range of concentrations, from 0.5-100 mM (Figure 7.2). At concentrations below 50 mM the gel did not form. When 50 mM calcium chloride solution was used, a loose gel structure began to form, but it was too weak to act as a cell growth scaffold. The gels formed easily at concentrations above 50 mM, but the gel could not then be disrupted later to isolate cells. These studies implied that we could not use alginate gel blocks as an interactive vesicle-gel for stem cell growth.

3.5 Neural Differentiation
To show that E14 stem cells could undergo selective differentiation when appropriate triggers were applied, neural differentiation using supplemented media was carried out. As the cells could not be extracted from the alginate gel blocks, the differentiation experiments were carried out on gelatin coated TCP. E14 cells were cultured in knockout DMEM supplemented with LIF to maintain pluripotency; these proliferated and formed colonies. These cells also exhibited round ES cell morphologies (Figure 7.3). E14 cells were also grown in N2B27 media containing nutrients required by neural cells with no LIF present. After 14 days these cells exhibited elongated cell morphologies and colonies which fan out from a central point. Cell attachment and morphology, although good indications, did not definitively show if differentiation had occurred. Immunofluorescence targeted specific proteins in cells which were used as markers to ascertain the state of the cell. Two of these were assayed; Oct IV is a homeodomain transcription factor that has a vital role in the self renewal process of undifferentiated stem cells, anti-neuron specific β-III tubulin targeted the β-III tubulin protein seen in E14 cells that have differentiated down the neural pathway, Figure 7.3. After 14 days E14 cells cultured in the presence of LIF showed Oct IV, demonstrating that the cells had remained pluripotent and did not show any β-III tubulin, which showed that no neural differentiation had occurred. In cells cultured in N2B27 neural media the reverse was true; no Oct IV was seen, but extended networks were seen with β-III tubulin indicating that complete differentiation had occurred and no pluripotent cells remained. The cells all appeared to be viable with structured nuclei visualised using DAPI.

3.6 Ascorbic acid triggered differentiation
For vesicle-nanoparticle triggered differentiation to occur, small single molecule triggers were needed. The neural differentiation model used an array of components to trigger differentiation and so were not easily encapsulated inside the vesicle cores. Ascorbic acid-2-phosphate was needed by chondrocyte cells for healthy growth. Ascorbic acid-2-phosphate has also been shown to be encapsulated and released easily from the vesicle-nanoparticle assemblies. We hypothesised that exposing stem cells to released ascorbic acid-2-phosphate could trigger chondrogenic
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differentiation. However, growing E14 cells with the addition of ascorbic acid-2-phosphate did not trigger differentiation (Figure 7.3). Light microscopy showed rounded E14 stem cells in colonies. Immunofluorescence showed that Oct IV was still present in the cells, confirming that the cells had maintained pluripotency.

3.7 Culture of E Cad\(^{(-/-)}\) cells
It has been demonstrated previously that stem cells which have the E Cadherin gene removed (E Cad\(^{(-/-)}\)) can be differentiated using simple triggers. E Cad\(^{(-/-)}\) cells have also been grown in the absence of LIF whilst remaining pluripotent. Initially when the cells were placed in media in the absence of LIF, cell death occurred. However further investigations showed that by decreasing the LIF gradually, the cells were maintained in its absence (Figure 7.4). E Cad\(^{(-/-)}\) cells cultured in media containing no LIF (X LIF media) showed the same morphologies as cells when LIF was present. E Cadherin controlled cell-cell contact. E Cad\(^{(-/-)}\) had no E Cadherin so the cells did not form colonies in either LIF or X LIF media and were observed as single cells not colonies in both media types (Figure 7.4). The cells did show similar rounded morphologies to E14 cells

3.8 Differentiation of E Cad\(^{(-/-)}\) cells
Ward et al reported that differentiation could be triggered in E Cad\(^{(-/-)}\) cells using simple molecules such as SB 431542 when the cells were cultured in the absence of LIF. As SB431542 is a simple molecule which could be encapsulated inside the vesicles and its release monitored by fluorescence it made it a suitable target for release studies (Figure 7.6). SB 431542 was encapsulated in vesicles by dissolving the SB 431542 in DMSO, which allowed mixing with PBS. This solution was then used to hydrate a lipid mixture of DPPC and biotinylated DHPE (0.2 % mol/mol). Extruding the lipid mixture 19 times through an 800 nm pore membrane gave vesicles with SB 431542 encapsulated inside. The vesicles were then aggregated with coated magnetic nanoparticles in the presence of avidin which gave large vesicle-nanoparticle aggregates as previously described (Section 2.11). These were seeded into hydrogel blocks (25 µL) suspended in PBS (1 mL), the gel blocks were placed in an alternating magnetic field the interactions between the magnetic nanoparticles and the changing field generated heat which triggered release of the SB 431542 from the vesicles (Figure 7.5k). Little spontaneous release from the vesicles was observed. Release of the SB 431542 was slow with little significant release seen until 120 minutes. This lag phase was probably due to the diffusion of SB 431542 through the alginate gel block. These data show that the vesicles were stable and SB 431542 was released into a biological system on the receipt of an alternating magnetic field trigger.
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3.9 Magnetically induced differentiation of E Cad$^{-/-}$ cells.

Vesicle-nanoparticle aggregates with SB 431542 encapsulated were investigated for their ability to trigger differentiation in E Cad$^{-/-}$ cells (Figure 7.5). Cells were cultured with vesicle-nanoparticle assemblies containing SB 431542 with and without magnetic release and with vesicle-nanoparticle assemblies containing PBS. The cells were cultured for 4 days in X LIF media on gelatin coated tissue culture plastic. Light microscopy showed little changes in the morphology between samples were SB 431542 is present and where it was absent making it unclear whether differentiation had occurred. The cells were still exhibiting rounded morphologies in all samples and no colonies had been formed. To understand if differentiation had occurred, immunofluorescence was used. Cell samples were stained for DAPI which enabled location of the cell nuclei and nanog which acted as a marker for pluripotency. Visualisation of the samples at 405 nm showed that the cell nuclei (blue) were present and intact in all samples. Nanog (green) seen at 488 nm was present in some samples indicating that differentiation had not occurred but absent in others. When SB 431542 was added directly to the cells, differentiation occurred and no nanog was seen. When vesicles with SB 431542 encapsulated, and released, using an alternating magnetic field, were cultured with the cells, differentiation also occurred and no nanog was present. When the cells were cultured with vesicles encapsulating SB 431542, but no release mechanism via the alternating magnetic field was used, some nanog was seen indicating that some differentiation had occurred. This would imply that the vesicles were not stable for 4 days in culture and some spontaneous leakage occurred in that time. When cells were cultured with vesicles containing PBS no differentiation occurred, suggesting that differentiation was solely due to the SB 431542 and not to the presence of vesicles. This proved that vesicles were used to store and release small molecules to trigger differentiation; however some leakage did occur in culture over an extended period of time. This initial data was encouraging as it suggested that liposomes were used to trigger differentiation in the stem cells. However, due to the fluctuating nature of nanog in stem cell population meant that ICC staining cannot be definitively used to elucidate whether differentiation has occurred. 

4 Conclusions

We have shown that surface based growth of embryonic stem cells on alginate gels was successful, although encapsulation of embryonic stem cells inside the gel matrix was a more challenging growth environment for the cells. This indicates that alginate based, interactive vesicle hydrogels used previously cannot be used for stem cell culture. Nevertheless this work demonstrates that vesicle-nanoparticle aggregates unsupported by a gel matrix can be used to encapsulate and release targeted differential triggers non-invasively. We conclude that this model for magnetically
targeted differentiation is of significant use in tissue engineering and merits further exploratory work. Our results have provided an insight into possible targeted drug delivery and differentiation processes.

Figures

Figure 7.1 E14 cells cultured on the surface of gels a) alginate, b) alginate doped with 1% fibronectin and c) gelatin coated tissue culture plate, E14 cells cultured inside gel blocks d) alginate, e) alginate doped with 1% fibronectin and f) gelatin suspension. g) Trypan blue cell exclusion assay of E14 cells on the surface of gel blocks alginate (diamond) alginate doped with 1% fibronectin (square) and gelatin coated tissue culture, h) trypan blue exclusion assay E14 cells encapsulated inside gel blocks. All scale bars are 100 µm and n=3.
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Figure 7.2  
(a) E14 cells after gel disruption with EDTA,  
(b) gel block after incubation with EDTA (10 mM),  
(c) gel block after incubation with EDTA (50 mM),  
(d) gel block after incubation with EDTA (100 mM),  
e) alginate gel solution after incubation with CaCl₂ (100 mM),  
f) alginate gel after incubation with CaCl₂ (50 mM),  
g) alginate gel solution after incubation with CaCl₂ (10 mM).

Figure 7.3  
E14 cells grown on gelatin coated tissue culture plastic in knockout DMEM doped with ascorbic acid-2-phosphate after  
(a) day 1,  
(b) day 3 and  
(c) day 5 stained with DAPI and Oct IV.  
d) ICC staining with OctIV and DAPI after 5 days.  
e) E14 cells cultured in knockout DMEM after 14 days,  
f) E14 cells cultured after 14 days in knockout DMEM, ICC staining with OctIV and DAPI,  
g) E14 cells cultured after 14 days in knockout DMEM, ICC staining with β-III tubulin and DAPI.  
h) E14 cells cultured in knockout DMEM after 14 days,  
i) E14 cells cultured after 14 days in N2B27 media, ICC staining with OctIV and DAPI,  
j) E14 cells cultured after 14 days in N2B27 media, ICC staining with β-III tubulin and DAPI.  
All scale bars are 100 µm, n=3.
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Figure 7.4 E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in media with a) LIF b) X LIF. All scale bars are 100 µm, n=3.

Figure 7.5 a) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media with vesicle-nanoparticle assemblies encapsulating PBS, b) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media with vesicle-nanoparticle assemblies encapsulating PBS, ICC stained using Nanog (green) and DAPI (blue), c) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media, d) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media, ICC staining using Nanog and DAPI, e) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media with vesicle-nanoparticle assemblies encapsulating SB 431542 with no release mechanism, f) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media with vesicle-nanoparticle assemblies encapsulating SB 431542 with no release mechanism, ICC staining using Nanog and DAPI, g) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media with SB 431542 added to the media, h) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media with SB 431542 added to the media, ICC staining Nanog and DAPI, i) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media with vesicle-nanoparticle assemblies encapsulating SB 431542 with alternating magnetic field pulse to trigger release, j) E Cad\textsuperscript{\textsuperscript{--/--}} cells cultured in X LIF media with vesicle-nanoparticle assemblies encapsulating SB 431542 with alternating magnetic field pulse to trigger release, ICC staining using Nanog and DAPI, k) magnetically triggered release of SB 431542 from vesicle-nanoparticle assemblies in alginate gel blocks. Scale bars are 100 µm.
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Figure 7.6 Structure of SB 431542
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Chapter 7
Magnetic Interactive Vesicle-Gels and their Uses as Cell Scaffolds
F de Cogan, JE Gough, SJ Webb

This chapter demonstrates preliminary data showing the uses and foibles of using magnetoliposomes for drug delivery.
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**Magnetic, interactive vesicle-gels and their uses as cell scaffolds.**  
Felicity de Cogan, Simon Webb and Julie Gough

**Abstract**

Creating biomaterials that replicate the complex structure and functions of tissue has so far proved to be challenging. The possible applications of such mimics are vast, ranging from subcutaneous drug delivery to tissue engineering. Furthermore the aggregation of vesicles (simple cell mimics) can give an insight into cell-cell adhesion. They can also act as active stores with drugs encapsulated, ready for controlled release. In this final chapter we look at the interactions between the release of encapsulated materials from liposome-nanoparticle aggregates and the alginate gel support. This chapter also reports release studies with MTT and glutamine which show good levels of release and poor levels of release respectively to demonstrate that the power of this novel delivery vehicle is in the choice of encapsulated material.

**1 Introduction**

Our previous work using phospholipid vesicles and biotin-coated magnetic nanoparticles showed that assemblies can be formed through biotin-avidin mediated aggregation. These assemblies were encapsulated inside alginate hydrogels, which in turn were used as cell scaffolds. This work showed successful interactions in this three dimensional structure. Biological cues were encapsulated and released magnetically, in order to gain a cell response. In the course of this work we identified areas which would benefit from further study: exploring experimental observations, such as variations in release rates, further biological release experiments and investigating what is required to create a successful, magnetically controlled vesicle-nanoparticle gels for cell scaffolds.

Phospholipid vesicles are a stable structure consisting of one or more concentric membranes and have an aqueous core, which can be used to encapsulate compounds of interest, such as drug molecules. The phospholipid bilayer encasing the encapsulated material has a number of phase states such as gel, liquid crystalline and lamellar. Phospholipid bilayers were modified either by covalently bonding interactive groups onto the phospholipid phosphate head or by attaching the molecule of interest to a lipid anchor, which held it in the membrane. Decorating the membrane of the vesicles with biotin encouraged aggregation and allowed them to act as cell mimics. The interaction of the surface groups on the bilayer also acted as an adhesive interface, which allowed the vesicles to communicate with their surroundings, instigating aggregate formation and cell attachment.

Fe$_3$O$_4$ magnetic nanoparticles have been studied extensively due to their size, biocompatibility and superparamagnetic nature. The nanoparticles are of special interest when interacting with an oscillating magnetic field, as they can be used to generate small amounts of localised heat *in situ.* Magnetic nanoparticles have uses in cell labelling and targeting, drug delivery, MRI and targeted hyperthermia. The nanoparticles were easily given an interactive surface through iron chelating groups, to make them dispersible in most
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solvents. These coatings were also targeted to specific applications such as cell internalisation as probes.

Although magnetic nanoparticles have been used as drug carriers, vesicles and magnetic nanoparticles are often used in tandem as magnetoliposomes. These provided a method by which a large store of active material in the vesicles was contained, controlled and then released magnetically. Magnetoliposomes have been very successful at delivering material non-invasively. Babincova et al demonstrated 90% release of encapsulated material using an alternating magnetic field to stimulate incorporated magnetic nanoparticles in vitro. In vivo studies by Zhu et al showed 50% release was obtained using magnetic fields. There survival of the vesicles in vivo was low, and they were often destroyed by cell internalisation. Encasing vesicle-nanoparticle aggregates in hydrogels stabilised the vesicle assemblies. Alginate gels are derived from algae and are therefore not easily recognised by the immune system in mammals, this allowed the gel to be used as a protective shield around the vesicle-nanoparticle aggregates.

Using alginate gels as a support for vesicles has been debated widely with conflicting reports arising on their suitability. It has been reported that the process of gelation, by the diffusion of calcium ions through the gel solution, caused gel fibres to penetrate the vesicle membrane. This penetration caused spontaneous leakage. As the gel became crosslinked, it expelled water from the system, which has also been linked to vesicle disruption. The character of the crosslinking agent reduced these unfavourable effects, e.g. barium ion gelation of alginate produced less spontaneous leakage than calcium ions. In other studies vesicles have been successfully incubated in alginate gels. In these studies no burst effect was seen at gelation and the vesicle aggregates were stable. It is possible that the relationship between the alginate gelation process and membrane disruption in the vesicles was lipid specific.

Using alginate as a cell scaffold has also been under debate. Extensive literature demonstrates the advantages and disadvantages of alginate as a biomaterial. Low cell attachment has been suggested as an advantage for alginate to be used as a bio bulking agent, as it is non-toxic, bioinert and does not promote biological interactions in vivo. Alginate can be crosslinked with calcium to form a stiff gel and so was often studied with chondrocyte cell lines rather than fibroblast cell lines. Cartilage cells were likely to be more stable in the gel than fibroblasts as they are less adherent and therefore are less dependent on the interactions with the cell scaffold. The cells in alginate show a rounded morphology with little attachment seen; this gave lower cell viability in fibroblasts but chondrocytes do not need such defined attachment. Alginate beads have been used as 3D scaffolds for stem cell culture.

In this report we investigate the interactions that form between vesicles and nanoparticles to create large assemblies and the effects that the surrounding environment had on assembly formation and disintegration. We also investigate alginate as a support for vesicle-nanoparticle aggregates and as a possible cell scaffold to create interactive gels. These ‘smart’ materials can be used as three dimensional scaffolds for cell growth, which can be manipulated externally in order to interact with the biological environment, using a non-
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An invasive trigger such as an external alternating magnetic field pulse. Studies showed that using an alternating field to induce nanoparticle mediated release was dependent on the interactions of the encapsulated material with the vesicles and the gels. The gel was only interactive with a biological system when the compounds encapsulated inside the system acted as a strong trigger.

2. Materials and Methods

2.1 Materials

DMPC, DPPC, biotin tagged DPPE and Rhodamine tagged DHPE were obtained from Avanti Lipids USA. Sodium alginate, 5/6-carboxyfluorescein, calcium chloride, 3-(N-morpholino)propanesulfonic acid, DMSO, Trypan Blue, resazurin sodium salt ‘amar blue’, bovine serum albumin (BSA), fluorescein isothiocyanate conjugated phallolidin (FITC-phallolidin), glutaraldehyde, dimethylthiазolylidiphenyltetrazolium bromide (MTT), paraformaldehyde (PFA), diamidinophenylindole (DAPI), Triton X-100, fluorescein isothiocyanate conjugated dextran (FITC-Dextran) nickel chloride, sodium hydroxide, biotin, N,N-Diisopropylethylamine (DIPEA), dimethylformamide (DMF), Calcein Blue and EDTA were all purchased from Sigma Aldrich, UK. L-Glutamine, penicillin/streptomycin, fetal bovine serum (FBS), trypsin-EDTA preparation, phosphate buffered saline (PBS) and Dulbecco’s modified Eagle’s medium (DMEM) F12 media were all obtained from PAA, Yeovil, UK. Tissue culture flasks, multiwall plates, ProLong Gold antifade reagent and Live/Dead assay, were all purchased from Invitrogen, Paisley, UK. 3T3 and C2C12 myoblast cells were obtained from the ECACC.

2.2 DPPC vesicles encapsulating 5/6-carboxyfluorescein

Liposomes were formed from DPPC (14.55 mg, 1.9x10^{-5} mol) and biotin-tagged DHPE (0.2 % mol/mol). The lipids were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. The lipid film formed was hydrated in 0.05 M 5/6-carboxyfluorescein in 3-(N-morpholino)propanesulfonic acid buffer at pH 7.4 (1 mL) and then extruded 19 times through an 800 nm polycarbonate membrane at 40 °C which gave vesicles of ~800 nm diameter encapsulating 5/6-carboxyfluorescein.

2.3 Formation of rhodamine-tagged vesicles

DPPC (14.55 mg, 1.9x10^{-5} mol) and biotin-DHPE (0.2 % mol/mol) were dissolved in a chloroform solution of rhodamine tagged DHPE (0.1 % mol/mol). The solvent was removed in vacuo and the lipid film dried under vacuum for 60 minutes. The film was then resuspended in PBS (1 mL) and the flask heated to 50°C and vortex mixed until the film was entirely resuspended. The lipid solution was extruded 19 times through an 800 nm polycarbonate membrane at 50 °C to give vesicles of ~800 nm diameter.
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2.4 Formation of Glutamine encapsulated liposomes

Liposomes were formed from DPPC (14.55 mg, 1.9x10^{-5} mol) and biotin-tagged DHPE (0.2 % mol/mol). The lipids were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. The lipid film formed was hydrated in a solution of glutamine in PBS (1 mL, 200 mM) and then extruded 19 times through an 800 nm polycarbonate membrane at 40 °C which gave vesicles of ~800 nm diameter encapsulating glutamine.

2.5 Formation of biotin coated magnetic nanoparticles

Iron (II) chloride tetrahydrate (0.546 g, 2.16 mmol) and iron (III) chloride hexahydrate (1.168 g, 4.32 mmol) were dissolved in deoxygenated water (5 mL). Sodium hydroxide (20 mL, 1 M) was added dropwise to the solution and stirred under nitrogen for 30 minutes. The solution was sedimented using neodymium boron magnet which precipitated the nanoparticles out of solution and the supernatant was removed. The particles were washed and resuspended in deoxygenated water (5 mL), then magnetically precipitated five times. The nanoparticles were dissolved in deoxygenated methanol (5 mL) and the solvent removed in vacuo, and stored under nitrogen.

The magnetic nanoparticles (25 mg) were suspended in deoxygenated methanol and N-biotinyl-(3,4-dihydroxyphenylethylamide) (6 mg, 1.58 x10^{-5} mol) was added. The mixture was sonicated for 3 hours under nitrogen. The coated nanoparticles were magnetically sedimented, using a NdBFe magnet. The sedimentsed nanoparticles were washed and resuspended in deoxygenated methanol, the repeated washing and magnetic sedimentation removed any non-chelated biotin-dopamine from the system. The solvent was removed in vacuo and the coated nanoparticles were stored under nitrogen.

2.6 Aggregation of vesicle nanoparticle assemblies.

DPPC, biotinylated-DHPE liposomes encapsulating 5/6-carboxyfluorescein were prepared as previously described (Section 2.2). The liposomes were aggregated with biotin coated magnetic nanoparticles (200 µL, 1.2 mg/mL) and avidin (10 µL, 10 mg/mL) for the following time periods, 30, 60, 120, 180, 240, 300, 360, 420, 1440 and 2880 minutes. At each time point the vesicle-nanoparticle assemblies were magnetically purified by magnetically sedimenting the aggregates, washing with PBS. The assemblies were resuspended in PBS supplemented with Triton X-100 (100 µL, 10 % vol/vol). The samples were then incubated at room temperature for 30 minutes and the solution aliquoted to a 96 well plate and the fluorescence read on Fluorostar Optima Plate reader at 520 nm after excitation at 460 nm.

2.7 Cell-vesicle gels imaged using environmental scanning electron microscopy

Vesicles were formed from DPPC and biotinylated DHPE (0.2 % mol/mol) as previously described (Section 2.2). The vesicle suspension was added to coated magnetic nanoparticles (200 µL of a 1.2 mg/mL) and avidin (50 µL of 10 mg/mL). The mixture was left to aggregate for 60 minutes. The vesicle aggregates were then magnetically sedimented using NdBFe
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magnet and the supernatant removed. The magnet was removed, the aggregates resuspended in PBS (1 mL), resedimented and washed 3 more times. After the last sedimentation the vesicle-nanoparticle aggregates were resuspended in sodium alginate (1 mL, 2 % wt/vol). This suspension (0.5 mL) was added to a 24 well plate. Calcium chloride (0.5 mL, 0.1 M) was added to each well and the gel left to cure for 30 minutes. The excess calcium chloride was removed and the gel block was washed with PBS (1 mL). 3T3 cell monolayer at 70 % confluency was separated using Trypsin.EDTA (0.105 mM, 3 mL) for 5 minutes at 37 °C and fresh media (2 mL) added. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant removed. The cell pellet was resuspended in fresh media (5 mL) and a sample (20 µL) transferred into a haemocytometer to ascertain a cell count. 3T3 cells (20,000) were then seeded on the top of each gel block and fresh media (0.5 mL) was added. The samples were incubated at 37 °C for 14 hours. The gel samples were washed with PBS (2 mL). The gel blocks were transferred to the plate of the scanning electron microscope and imaged without stains or coatings. Samples were run on a Zeiss Evo 60 Extended Pressure scanning electron microscope.

2.8 Encapsulation of 2-aminoacridone labelled heparin sulphate in vesicle gels.

2-Aminoacridone labelled heparin sulphate was prepared from freeze dried heparin sulphate. The heparin sulphate was dissolved in heparinase buffer (100 µL) and heparinase III (5 µL) was added and the sample was vortexed and incubated at room temperature for 4 hours. A second heparinase aliquot (5 µL) was added, the sample was vortexed and incubated overnight at room temperature. The sample was then freeze dried. 2-Aminoacridone (10 µL, 0.1 M in DMSO/acetic acid) was added to the digested heparin sulphate and the sample incubated at room temperature for 20 minutes. Sodium cyanoboro hydride (10 µL, 1 M) was added to sample and the sample was vortex mixed. The mixture was incubated overnight at room temperature. The freeze dried AMAC-heparan sulphate was dissolved in PBS (1 mL). Liposomes were formed from DPPC (14.55 mg, 1.9x10^-9 mol) and biotin tagged DHPE (0.2 % mol/mol). The lipids were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. The lipid film formed was hydrated in AMAC-heparan sulphate solution (25 µL of AMAC-labelled heparin sulphate in 975 µL of PBS) and then extruded 19 times through an 800 nm polycarbonate membrane at 50 °C to give vesicles of ~800 nm diameter. The vesicles were aggregated with magnetic nanoparticles using avidin and suspended in vesicle gels as described previously (Section 2.6).

2.9 Release of 2-aminoacridone labelled heparin sulphate from vesicle gels.

Vesicle gels were formed using vesicles encapsulating 2-aminoacridone labelled heparin sulphate in a 5x5x1 mm alginate gel block (Section 2.8). The gel block was suspended in PBS (2 mL). The sample was then exposed to an AMF (392 kHz) for 240 s, then aliquots of the solution (200 µL) were taken every 60 minutes. The fluorescence emission of the released AMAC-heparan sulphate was monitored at 485 nm after excitation at 420 nm. Thermal release was obtained by heating the sample to 50 °C for 4 minutes before the solution fluorescence was measured.
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2.10 Encapsulation of (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in vesicle gels.
Liposomes were formed from DPPC (14.55 mg, 1.9x10^{-5} mol) and biotin tagged DHPE (0.2 % mol/mol). The lipids were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure. The lipid film formed was hydrated in a solution of MTT in PBS (2.4 mM, 1 mL) and then extruded 19 times through an 800 nm polycarbonate membrane at 50 °C to give vesicles with an ~800 nm diameter. The vesicles were aggregated with magnetic nanoparticles using avidin and suspended in alginate gels as described previously (Section 2.6).

2.11 Release of MTT from vesicle gels
Vesicle gels were formed using vesicles encapsulating MTT in a 5x5x1 mm alginate gel block (Section 2.10). The gel block was suspended in PBS (2 mL). The sample was then exposed to an AMF (392 kHz) for 240 s, then aliquots of the solution (200 µL) were removed every 60 minutes. The fluorescence emission of the released MTT in these aliquots was monitored at 540 nm. Thermal release was obtained by heating the cuvette to 50 °C for 4 minutes before fluorescence was measured.

2.12 Myoblast cell growth on glutamine vesicle gels
Myoblast cells were harvested and centrifuged at 1500 rpm for 5 minutes. The supernatant removed and the cells were resuspended in fresh media. The cells were then added to sodium alginate solution or sodium alginate solution containing L-glutamine encapsulated in vesicle-nanoparticle assemblies at a density of 20,000 per gel. The cell-alginate suspension was added to glass culture well (0.5 mL) and cured using calcium chloride (0.5 mL, 100 mM). The excess solution was removed and the gels were washed with PBS. The gels with no vesicles present were incubated in media with L-glutamine present and media without L-glutamine present. The gels with vesicles present were incubated in media without L-glutamine present and either did or did not undergo an alternating magnetic field pulse. The plates were incubated at 37 °C and CO₂ (5 % vol/vol) and alamar blue and DNA assays were completed every 24 hours for 5 days. Alamar blue assays were carried out by removing the media surrounding the gel block. The gel block was washed with PBS (2 mL) and resuspended in fresh media (1 mL) and alamar blue solution (100 µL, 150 µM) was added. The samples were incubated at 37 °C, CO₂ (5 % vol/vol) for two hours. Aliquots of the media surrounding the gel were transferred into a 96 well plate (200 µL/well) and the fluorescence read at 590 nm following excitation at 560 nm. The gel blocks were washed with PBS (2 mL), suspended in distilled water (1 mL) and frozen at -80 °C for 48 hours in preparation for the DNA assay. The DNA assay was run by thawing the samples to room temperature, refreezing them at -80 °C and rethawing them repeatedly. After the plates had been frozen and defrosted three times the solution in the plates was then added to a 96 well clear bottom plate in 3 aliquots (50 µL) from each well. TNE buffer (50 µL) was added to each well of the 96 well plate and Hoescht 33342 stain (100 µL) was also added. Fluorescence was then read at 460 nm following excitation at 420 nm.
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2.13 MTT encapsulated in vesicle gels as cell scaffolds.

MTT encapsulated in liposomes was prepared as previously described. The vesicles were aggregated with magnetic nanoparticles (200 µL, 1.2 mg/mL) and avidin (10 µL, 10 mg/mL) for 60 minutes. The aggregates were magnetically purified by magnetic sedimentation and washing the sedimented material followed by resuspension in sodium alginate solution (2 % wt/vol, 1 mL). 3T3 cells were harvested and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the cells were resuspended in fresh media. Sodium alginate and alginate-vesicle solutions (0.5 mL) were added to glass culture wells and cells were seeded into the solutions at a density of 40,000 per well. The mixtures were cured using calcium chloride (0.1 M, 0.5 mL), then the excess solution was removed by pipette and the gels washed with PBS. The gels were resuspended in fresh media (1 mL) and vesicle samples were pulsed using an alternating magentic field (392 kHz, 240 s). Calcium alginate gels with no vesicles present had MTT (100 µL, 1 mg/mL) added to the media. The samples were incubated at 37 °C and CO₂ (5 % vol/vol). At 60 minute timepoints the media was removed from a sample and the gel was resuspended in isopropyl alcohol (1 mL) and shaken for 60 minutes. The isopropyl alcohol was then removed and the absorbance of the solution was read at 540 nm.

2.14 Myoblast cell culture

2.14.1 Cell media

All myoblast cell experiments were cultured in Dulbecco’s Modified Eagle Media (DMEM) with the addition of Fetal Bovine Serum (10 % vol/vol, 50 mL) and Penicillin/streptomycin antibiotics (1 % vol/vol, 5 mL) unless otherwise specified. The media was stored at 4 °C and warmed to 37 °C before use.

2.14.2 Cell culture

Frozen cell suspensions were stored over liquid nitrogen in fibroblast media with 10 % vol/vol DMSO (1 mL) were removed from the liquid nitrogen, vented and warmed to 37 °C. The cell suspension was then added to cell media (4 mL) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed leaving a cell pellet which was resuspended in media (10 mL) and added to a T75 culture flask. The media on the flask was changed every 48 hours and the flask was split when cell confluence reached 70-80 %.

The media was removed from the flask and the cell monolayer washed twice with PBS. Trypsin.EDTA (0.105 mM, 3 mL) was added to the flask which was incubated at 37 °C for 5 minutes. Media (2 mL) was then added to the flask and pipetted to ensure removal of the cell monolayer from the flask, and the cells centrifuged at 1500 rpm for 5 minutes. The cells were then resuspended in fresh media (10 mL), split in a 1:3 ratio and added to new flasks with fresh media (7 mL).

Cryostore of cells was obtained by removing the media from a confluent flask and washing twice with PBS. Trypsin.EDTA (0.105 mM, 3 mL) was added to the flask which was incubated for 5 minutes. Media (2 mL) was then added to the flask and the cells centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the cell pellet was then
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resuspended in media (10 mL) with DMSO (10 % vol/vol, 1 mL), aliquoted into cryovials (1 mL) and placed in a Mr Frosty at -80 °C for 48 hours and then transferred to liquid nitrogen.

2.15 3T3 cell culture
2.15.1 Cell media
All 3T3 fibroblast cell experiments were cultured in Dulbecco’s Modified Eagle Media (DMEM) with the addition of Fetal Bovine Serum (10 % vol/vol, 50 mL) and Penicillin/streptomycin antibiotics (1 % vol/vol, 5 mL). The media was stored at 4 °C and warmed to 37 °C before use.

2.15.2 Cell culture
Frozen cell suspension stored over liquid nitrogen in fibroblast media with 10 % DMSO (1 mL) were removed from the liquid nitrogen, vented and warmed to 37 °C. The cell suspension was then added to cell media (4 mL) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed leaving a cell pellet which was resuspended in media (10 mL) and added to a T75 culture flask. The media on the flask was changed every 48 hours and the flask was split when cell confluency reached 70-80 %.

Cryostore of cells was obtained by removing the media from a confluent flask and washing twice with PBS. Trypsin.EDTA (0.105 mM, 3 mL) was added to the flask which was incubated at 37 °C for 5 minutes. Fresh media (2 mL) was then added to the flask and pipetted to ensure removal of the cell monolayer from the flask surface, and the cells centrifuged at 1500 rpm for 5 minutes. The cells were then resuspended in fresh media (10 mL), split in a 1:3 ratio and added to new flasks with fresh media (7 mL).

2.16 Cell-vesicle interactions.
Cells were obtained by removing the media from a confluent flask and washing twice with PBS. Trypsin.EDTA (0.105 mM, 3 mL) was added to the flask which was incubated for 5 minutes. Fresh media (2 mL) was then added to the flask and the cells centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the cell pellet was then resuspended in media (10 mL) with DMSO (10 % vol/vol, 1 mL), aliquoted into cryovials (1 mL) and placed in a Mr Frosty at -80 °C for 48 hours and then transferred to liquid nitrogen.
3. Results and Discussion

3.1 Formation of vesicle-nanoparticle aggregates.

Biotinylated phospholipid vesicles aggregate with magnetic nanoparticles through interactions with avidin. Avidin is a tetrameric protein which forms strong interactions with up to four biotin groups at a time. Doping the DPPC phospholipid membranes with biotinylated DHPE (0.2% mol/mol) gives an interactive layer of biotin on the surface of the vesicles. Magnetic nanoparticles were formed by co-precipitation and coated through biotin-dopamine ligand. The ligand has two hydroxyl groups on the benzene ring which chelate to the iron present in the nanoparticles (Figure 8.1a). On the addition of avidin solution, the avidin protein crosslinks with the biotin layers on both the vesicles and the nanoparticles, creating large aggregates. The aggregates were suspended in alginate, which gave interactive gels, which were used as cell scaffolds. The amount of the aggregated material related directly to the time allowed for the vesicles and nanoparticles to aggregate (Figure 8.1d). Higher levels of release were seen from structures which have a longer aggregation time. Vesicles were formed with 5/6-carboxyfluorescein encapsulated, in order to allow visualisation with fluorescence microscopy. The longer the aggregates had to form, the larger the assemblies that were observed (Figure 8.1 e-g). Quantitative studies were carried out showing aggregation times between 0.5 to 48 hours. These showed that when larger assemblies were observed higher levels of carboxyfluorescein release were obtained, when the vesicles in the assemblies were released using Triton X-100. The assemblies were purified magnetically and resuspended in PBS. The contents of the vesicles were released using Triton X-100. Non-aggregated liposomes were removed during magnetic sedimentation and washing so only aggregated liposomes released the 5/6-carboxyfluorescein. The amount of 5/6-carboxyfluorescein released from the vesicles was time-dependent on the time allowed for vesicle-nanoparticle aggregation. This demonstrated that a higher level of aggregation occurred when a longer space of time was allowed. The amount of 5/6-carboxyfluorescein released from assemblies grew over the first 4 hours as the vesicle nanoparticle aggregates formed. Then little change was observed over the next 20 hours. This was attributed to a maximum aggregation point for which several possible factors could be suggested, such as spatial constraints, concentration dependence and diffusion. At 48 hours the amount released from aggregated vesicles had decreased, suggesting that after the 24 hour peak, the aggregates were not stable in solution and began to degrade. In order to stabilise the aggregates, they were supported in an alginate gel matrix. Scanning electron microscopy was used to image the individual vesicles in the assemblies to detect if they were stable through the gelation process (Figure 8.1h). This showed that the vesicles were intact and the gel fibres did not penetrate the vesicles, causing membrane disruption. Fluorescence studies into the stability of the vesicles in the alginate gels showed that the gelation process of the alginate did not cause membrane disruption, no spontaneous leakage was observed during incubation and vesicle aggregates were seen spread throughout the gel (Figure 8.1e-g).
3.2 The interactions of vesicles and nanoparticles with cells.

To determine the suitability of vesicle nanoparticle aggregates for biological processes, the interactions of the vesicles and nanoparticles with cells was studied. 3T3 fibroblast cells were used as they are robust adherent cell line. Cells were cultured in the presence of magnetic nanoparticles in order to measure any toxic effects or any retardation of cell proliferation. Cells were then cultured with magnetic nanoparticles added to the media and held in close proximity to the cells magnetically, by placing the well plate in a magnetic field. Preliminary data showed that the magnetic nanoparticles did not appear to have any adverse effects on the fibroblast cells. Preliminary data showed no decrease in proliferation observed and cell morphologies remained comparable to control samples. Interestingly when cells were cultured on a magnet, the cell number decreased, whether nanoparticles were present or not (Figure 8.3). This would suggests that although magnetic nanoparticles and oscillating magnetic fields do not affect cell monolayers, long term exposure to a strong, permanent magnets could.

3.3 Release of encapsulated material using an alternating magnetic field pulse

When vesicles were aggregated with magnetic nanoparticles, an alternating magnetic field could be used to trigger release. The length of time for which the vesicle-nanoparticle aggregates were exposed to the field did affect the rate of release, but not the final release amount, which remained constant (Figure 8.2a). 5/6-carboxyfluorescein was encapsulated inside vesicles and then aggregated with magnetic nanoparticles. These assemblies were suspended in an alginate gel block. When the gel was pulsed for 2 minutes, after a 10 minute incubation period at room temperature, release was gradual and full release was not observed until 40 minutes. When a single 4 minute pulse was used, release was still a gradual process, with full release reached at 40 minutes, but the initial release was slightly faster than when a single 2 minute pulse was used. When samples were subjected to a 2 minute long pulse, every 5 minutes throughout, full release was obtained much earlier at 20 minutes. Step increases were observed, where there were sharp increases in fluorescence at each pulse. This was mirrored when a 4 minute pulse was used. This increase in the rate of release was due to the localised heat generated by the magnetic nanoparticles as they constantly realigned with the changing field, speeding up release from the vesicles. The lag time observed was the diffusion time through the alginate gel matrix. Carrying out further studies on 5/6-carboxyfluorescein encapsulating vesicles displayed interesting results. When magnetic nanoparticles and an alternating magnetic field were used to trigger release, different amounts of release occurred (Figure 8.2b). Magnetically triggered release varied from 40 to 90 %. These variations seemed to be dependent on the magnetic nanoparticles, with age, coating efficiency and atmospheric conditions at formation all affecting the amount of 5/6-carboxyfluorescein released. Also observed in these experiments was that spontaneous release sometimes occurred at gelation. Although most experiments showed no spontaneous release, several occurrences were seen. This is likely to be the result of penetration of the vesicle membrane by the alginate prior to gelation and as has been documented by Langer et al. The exact causes of the lack of reproducibility are unknown. To gain substantial data for cell and release experiments, each batch of nanoparticles was tested after synthesis to
ascertain if they were suitable for release experiments. If they were suitable high levels of release were observed. When low release was observed with the nanoparticles the materials were disposed of and a new batch synthesised.

The phospholipid vesicles interacted well with the magnetic nanoparticles and were stable under physiological conditions. When the vesicles were cultured in the absence of magnetic nanoparticles and avidin they still formed aggregates. These were formed through loose interactions between the vesicles and disintegrated when subjected to agitation (Figure 8.4). These aggregates formed by charge interactions, which also enabled the vesicles to interact with the cells and allowed them to adhere to the cell surface. The concentration of the vesicles and the cells was related to either the formation of vesicle aggregates or the formation of vesicle/cell assemblies. At high vesicle/low cell concentrations vesicle aggregates formed and little interaction with the cell monolayer was observed. At low vesicle/ high cell concentrations vesicles were observed interacting with the cell membrane in preference to other vesicles. The vesicles were incubated with the cells, for 24, 48 and 72 hours, the samples were imaged and experimental observation showed vesicle motion upon agitation so the vesicles were not internalised by the cells.

3.4 Interactions of released compounds with the biomaterial
To create an interactive material for use in a biological environment, the vesicles needed to encapsulate bioactive compounds. The linear polysaccharide heparan sulphate binds a variety of proteins and growth factors and is vital to the regulation of biological processes such as embryogenesis and tumor metastasis. It also has roles in elucidating disease mechanisms. Vesicles were formed with 2-aminoacridone (AMAC)-labelled heparin sulphate encapsulated. Heparan sulphate was partially digested enzymatically and labelled fluorescently using AMAC. A lipid film formed as previously described (Section 2.8) was resuspended in the heparin sulphate solution and extruded 19 times through an 800 nm pore membrane. The vesicles were stable in alginate gels with no spontaneous leakage observed (Figure 8.5a). Release of the heparan sulphate was obtained using an alternating magnetic field which generated full release after 2 hours.

3.5 Vesicle gels as in vitro cell monitors
MTT is used as a cell assay to determine the metabolic state of living cells. The tetrazole is reduced to formazan by reductase enzymes present in the cells. Studies into encapsulating MTT inside vesicle gels and its release into the biological environment are of interest on many levels. Previous work has shown that Ni²⁺ ions take longer to pass through alginate gels than large dye molecules such as 5/6-carboxyfluorescein. This was due to the electrostatic interactions of the nickel ions with the gel matrix. MTT carries a positive charge and could be used to ascertain the nature of the interaction between charged species and alginate. MTT is also used as a test for viable cells. Vesicle gels which encapsulate and release MTT can be self monitoring, by detecting any changes to cells in the vesicle gel internally and non-invasively during experiments. This reduces the need for the destruction of samples when a
Chapter 7 Magnetically Interactive Vesicle Gels and their uses as Cell Scaffolds

possible change has occurred. Vesicles were formed by dissolving DPPC and biotinylated DHPE (0.2 % mol/mol) in chloroform and the lipid film was dried \textit{in vacuo}. The lipid mixture was then resuspended in MTT in PBS and extruded 19 times, through an 800 nm pore membrane. The vesicles with MTT encapsulated were stable in the alginate gel, with no spontaneous leakage observed (Figure 8.5b). The MTT was released fully using an alternating magnetic field. Full release was obtained after 4 hours. The lag phase for the MTT was due to interactions between the MTT and the alginate gel. Comparison of released material, based on size and charge, showed that strong interactions hindered the passage of released material through the alginate gel blocks (Figure 8.6a). This showed that the fastest release occurred from 5/6-carboxyfluorescein.

5/6-carboxyfluorescein is a small neutral molecule and so will have little interaction with the gel matrix. Full release was observed in 20 minutes; this lag phase was due to diffusion processes. AMAC-labelled heparin sulphate also showed a fast release time with full release obtained in 2 hours. AMAC-labelled heparin sulphate has little electrostatic interaction with the gel, but the large size of the released material slowed down diffusion. When charged species such as nickel chloride and MTT were used, release became much slower. Size played an important role in release again, interestingly MTT, which is charged, but has a much larger size than Ni\(^{2+}\) ions, diffused faster than the nickel ions. The retardation of the nickel by the gel was explained, as nickel ions have similar spatial and charge dimensions to Ca\(^{2+}\) ions. Ca\(^{2+}\) ions are diffused through alginate solutions and are chelated strongly between the alginate strands, forming the gel network. Nickel chloride fits between the alginate strands and gets ‘locked’ into the matrix in the place of Ca\(^{2+}\) therefore exhibiting slow release profiles. MTT did interact strongly with the gel due to its cationic nature. However, it could not slot into the strands as both nickel and calcium ions did, so ultimately was released faster despite its larger size.

3.4 Interactive vesicle gels as cell scaffolds

MTT vesicles gels can act as interactive cell scaffolds. The alginate gel provides a 3 dimensional environment. The vesicles, when release is triggered, showed that the MTT diffused through the gel and interacted with the cells present. 3T3 cells were cultured on vesicle gels, as the fast metabolism of the cells, allowed easy visualisation of the metabolism of MTT. The release and metabolism of MTT was monitored fluorescently as the tetrazole MTT structure is yellow absorbing at 520 nm. After MTT has been reduced using reductase enzymes, it forms formazan which is purple and absorbs at 450 nm, allowing the system to be monitored after the reduction (Figure 8.6).

When cells were cultured in alginate gels with no vesicles present, but MTT solution was added to the cells, the MTT was metabolised and a colour change was observed. This indicated that the MTT was capable of diffusing through the gel block. It also demonstrated that the cells were live and viable inside the vesicle gel. Cells were grown in vesicle gels, held in close proximity to vesicle-nanoparticle aggregates. The gels were placed in an alternating magnetic field; again a colour change was observed. This proved that the vesicles used to encapsulate the MTT successfully released it to interact with the cells. Cells were grown on vesicle gels where MTT was encapsulated inside vesicles. The vesicles were held
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in close proximity to the cells, but no alternating magnetic field was used to trigger release. No colour changes were observed in this sample. Without the trigger to cause release from the vesicles, no MTT was free to be metabolised, inducing a colour change. Negative controls were also carried out with neither MTT nor vesicles encapsulating MTT present. In these controls no colour change was observed. Vesicle gel experiments have not always been successful. The creation of interactive gels is highly dependent on the material used for encapsulation or release.

Glutamine is required for cell growth as it is a vehicle for the transfer of α-amine nitrogen and is an energy source for rapidly growing cells. Myoblast cells grow and metabolise at a very fast rate; therefore glutamine is vital for their cell proliferation. In an attempt to tap into the process for which the cell utilises glutamine, vesicle gels were created where glutamine was encapsulated inside vesicles and released in order to ascertain any effects on cell proliferation or metabolism. Myoblast cells grew well on alginate gels (Figure 8.7a-f). Cells were cultured in gels with and without glutamine present and also with vesicle containing glutamine with and without release. No relation was discoverable between the presence of glutamine and the level of cell proliferation, although cells cultured in gels where vesicles were present (whether glutamine was released using an alternating magnetic field or not) showed slightly higher cell numbers. When the cell metabolism was studied it was observed that once again the presence of glutamine had little effect at all. Some data correlations showed that samples showed a higher metabolic rate in the absence of glutamine. This has demonstrated that although vesicle gels are a highly innovative tool for possible drug delivery methods, the usefulness of the gels is dependent on the material chosen and released from the vesicles.

4 Conclusions
In this study we have looked closer at some of the processes that occur when using vesicle gels as cell scaffolds. The interactions between vesicles, magnetic nanoparticles and cells have been demonstrated to be harmless. Although variations in the rates of release are observed when using magnetic nanoparticles and as oscillating magnetic field to trigger release, release does always occur. The interactions of the released material and the surrounding hydrogel have transpired to be the strongest influence on the release profile, as the polyanionic gel interacts with cationic released material. Vesicle gels which have successfully interacted with a biological environment (MTT) and unsuccessfully (glutamine) have been shown here, to demonstrate that the efficacy of these vesicle gels is dependent on the interactions between the cells and the biologically active compounds released.
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Figures

Figure 8.1 a) Synthetic biotin-dopamine linker chelated to Fe₃O₄ magnetic nanoparticle, b) the biotin tagged lipid anchor in the vesicle phospholipid bilayer. c) Avidin mediated aggregation of DPPC vesicles and coated magnetic nanoparticles d) 5/6-Carboxyfluorescein release after aggregation at each time point, normalised to maximum release. e-g) vesicles encapsulating 5/6-carboxyfluorescein spread throughout the gel matrix, after aggregation time of e) 30 minutes, f) 60 minutes and g) 120 minutes all scale bars show 1 µm. h) vesicle gels imaged using AFM i) dehydrated, sputter coated vesicle gels imaged using SEM, scale bar is 1 µm.

Figure 8.2 a) Magnetic release of 5/6-carboxyfluorescein from vesicle gels with repeated 4 minute 392 kHz pulse every 5 minutes (X), repeated 2 minute 392 kHz pulse every 5 minutes (triangle), one two minute 392 kHz pulse at 10 minutes (diamond), one four minute 392 kHz pulse at 10 minutes (square). b) Release of 5/6-carboxyfluorescein from vesicle gels release using 50 °C thermal stimuli for 4 minutes (green) release using a 392 kHz magnetic pulse (red), no release stimuli (blue).
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**Figure 8.3** Cell growth of 3T3 cells cultured with magnetic nanoparticles (black) and without magnetic nanoparticles (white), n=3.

**Figure 8.4** Cell vesicle interactions a-f) after 24 hours, g-l) after 48 hours and m-r) after 72 hours in standard 3T3 cell culture.
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Figure 8.5 a) Magnetic release of AMAC-labeled heparin sulfate from vesicle gels using 392 kHz magnetic pulse (diamond) no pulse (square) measured at 485 nm, n=3. b) Magnetic release of MTT from vesicle gels using 392 kHz magnetic pulse (triangle) no pulse (square) measured at 540 nm, n=3.

Figure 8.6 a) Magnetic release of 5/6-carboxyfluorescein (square), AMAC-labelled heparin sulfate (triangle), MTT (diamond) and nickel ions (X), all using a 392 kHz pulse, n=3. b) MTT released from vesicle-nanoparticle aggregates using a magnetic pulse and reduced by 3T3 cells (diamond), MTT encapsulated in vesicles with no stimuli to induce release and cultured with 3T3 cells (triangle), 3T3 cells cultured on alginate gels with no MTT present (square), n=3.
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Figure 8.7 a-f) rhodamine tagged vesicle-nanoparticle aggregates (red) and myoblasts grown on alginate gels, nuclei (DAPI-Blue), actin cytoskeleton (FITC-Phalloidin-green). g) metabolic activity of myoblast cells and h) cell growth of myoblast cells when cultured with glutamine (triangle), no glutamine (X), vesicles encapsulating glutamine (diamond) and vesicles encapsulating glutamine with a 392 kHz pulse to trigger release (square).


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Chapter 8

Conclusions and Future Work

This chapter draws conclusions from all of the work in this thesis both published and unpublished and suggests some of the future work on this project.
Chapter 9 Conclusions and future work

8.1 Conclusions

The main aim of this thesis was to build an interactive cell scaffold which could release biologically active drugs on the receipt of a magnetic trigger. This work was built on initial studies carried out in the Webb group, creating vesicle-magnetic nanoparticles constructs which could release encapsulated material when placed in an alternating magnetic field. As anticipated forming a fully interactive biomaterial from this work required extensive system modification. This thesis examined two processes. Firstly the chemistry used to create the vesicle gels and secondly the interaction of the vesicle gels in biological systems.

The synthesis of biotin tagged ligands demonstrated in this work allowed the formation of synthetic Fe$_3$O$_4$ nanoparticles with an interactive layer of biotin which interacted with the external environment. When these were mixed with liposomes, with biotin anchored lipids incorporated into the membrane in the presence of avidin, we have demonstrated that aggregation did occur. Liposomes formed from DPPC were stable during formation and aggregation with magnetic nanoparticles, while a range of different compounds was encapsulated. The liposome-nanoparticle aggregates released the encapsulated material when placed in an alternating magnetic field.

Liposome-nanoparticle assemblies showed interactions with a range of possible hydrogel supports. The liposomes had varying stability in the different gels and the most suitable gel was found to be alginate. Although some literature reported seeing burst effects when liposomes were held in alginate gels, this was not observed in this work.

Primary cells and cells from established cell lines were studied on different hydrogels to ascertain the best cell scaffold for the vesicle-gels. Our studies showed that although cell proliferation occurred, it was at a low rate on alginate gels. Low attachment was overcome by the addition of fibronectin to the gel matrix. Although cells and liposome-nanoparticle assemblies were encapsulated separately in the gel matrix, interactions between the liposomes and the cells occurred, with strong adhesions forming between the liposome and the cell membranes. Unlike previous
Chapter 9 Conclusions and future work

literature reports the liposomes were clearly binding to the cell surface and were not internalised by the cells.

The interactive vesicle-gels were shown to release the encapsulated material held inside the liposomes easily when placed in an alternating magnetic field. The release of the material was dependent on the interactions with the gel matrix. Positively charged species such as Ni$^{2+}$ and MTT showed a longer lag phase as electrostatic interactions with the polyionic gel hindered their diffusion through the gel. The release of nickel ions was particularly delayed as the Ni$^{2+}$ ions were thought to chelate into the Ca$^{2+}$ binding sites which crosslinked the gel network.

The release of encapsulated material from liposome-nanoparticle assemblies as the gel was used as a cell scaffold showed that the vesicle gels were interacting with the cell population. The encapsulation and release of ascorbic acid-2-phosphate triggered collagen production in chondrocytes. The release of nickel chloride caused cell death in 3T3 cells and the release of ascorbic acid-2-phosphate resulted in collagen deposition by bovine chondrocytes.

When the vesicle gels were moved from cell culture to embryonic stem cell culture our investigations showed that culture of pluripotent embryonic stem cells on alginate vesicle gels was possible. However, encapsulation of the stem cells inside a large gel block was not a viable option due to decreased cell attachment, proliferation and the formation of embryoid bodies. Stem cell culture using free floating vesicle-nanoparticle aggregates was possible and resulted in magnetically triggered differentiation.

Magnetoliposomes have been used extensively as drug delivery agents both *in vitro* and *in vivo*. One of the main challenges is in incorporating stimuli sensitive magnetic nanoparticles into the liposome structure. Methods of encapsulating them in the aqueous core$^{60}$ or embedding them in the phospholipid bilayer$^{61}$ both hinder efficiency. The work in this thesis has presented an alternative form of magnetoliposome that addresses this problem and, unlike previous work, is stable and interactive in a biological system.$^{62}$
Chapter 9 Conclusions and future work

Limitations still remain in this work. The liposomes used show low encapsulation efficiency, and although magnetic targeting is possible of a large area, precise targeting in vitro could not be attained. Literature studies have shown far more precise methods of targeting, such as incorporating antibodies into the liposome membrane, which directs the liposomes in both a region- and a cell-specific manner.\(^1\)

However, the work in this thesis has shown that we have created novel biomaterials which can deliver bioactive compounds. The transparent nature of mammalian tissue to magnetic fields allows the released cues to be non-invasive. We have created a novel ferrobiomaterial which can be externally controlled both spatially and temporally.

8.2 Future work

Further work is necessary on this project to allow it to progress towards clinical application. This work would include in depth, controlled release studies utilising the magnetic nanoparticle – alternating magnetic field response. Our work has shown that magnetic release of encapsulated material can vary from 40 to 90 %. This variation appeared to be dependent on the magnetic nanoparticle formation conditions. The causes of these variations need to be discovered and fully quantified.

Immediate future work would include using liposomes formed from lipids which demonstrate phase transitions at different temperatures. This would allow the encapsulation of several active compounds in different liposomes inside the cell scaffold. Each set of liposomes would release their encapsulated material on the receipt of a magnetic trigger of different lengths.

Also included in this work are initial experiments which suggest that although magnetic nanoparticles alone had no effect on cell viability, culture of cells on a strong, permanent magnet could produce low levels of cytotoxicity. This is an interesting observation although not in the scope of this work merits further investigation.
Chapter 9 Conclusions and future work

Long term future plans for the project would be to encapsulate specific materials for release in an *in vivo* disease model. One possibility would be to encapsulate cancer drugs such as doxorubicin, which would allow the magnetoliposomes to deliver targeted release to cancer cells. This would also offer the opportunity to test the novel magnetoliposomes against current treatments used in patients.

Initial work with E14 and E Cad$^{(-)}$ cells showed that the liposome-nanoparticle assemblies could be used to encapsulate and release stem cell differentiation triggers. When no release trigger was in place some spontaneous differentiation did occur. Further investigations to maintain the stability of the liposomes in stem cell culture to prevent spontaneous leakage and providing a scaffold which could both support the liposome nanoparticle aggregates and act as a cell scaffold would be essential for future work in this area.
Chapter 9 Conclusions and future work

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Appendix

Appendix

This paper was an invited publication to the Proceedings of the Material Research Society following presentation of the work at the MRS conference. This work showed the preliminary data carried out at the time, but the following areas require addressing following further investigations reported in this thesis.

1. In the experimental details sodium alginate was used not alginic acid.
2. The graphs of the 3T3 cell lines showing the data from the alamar blue assay are adjusted using the standard curve and therefore show the cell number not total metabolic activity.
3. The morphology data referred to in the text is not shown in this publication although it is seen in other areas of this thesis.
4. The graphs showing the data for the metabolic activity (alamar blue assay) with chondrocyte cells are adjusted using a standard curve and therefore show cell number not metabolic activity.
Appendix

Enhancing cell culture in magnetic vesicle gels

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ABSTRACT

Several different hydrogel compositions have been incorporated into magnetic vesicle gels and the resulting “smart” biomaterials assessed as cell culture scaffolds. The compatibility of these hydrogels with the “smart” component of these biomaterials, thermally sensitive vesicles (TSVs) crosslinked by magnetic nanoparticles, was assessed by the leakage of fluorescent 5/6-carboxyfluorescein from the TSVs under cell culture conditions. Subsequently the ability of the hydrogels to support 3T3 fibroblast and chondrocyte viability was assessed. These studies revealed that alginate-based gels were the most compatible with both the TSVs and the cultured cells, with an alginate:fibronectin mix proving to be the most versatile. Nonetheless these studies also suggest that TSV composition needs to be modified to improve the performance of these “smart” cell culture scaffolds in future applications.

INTRODUCTION

Creating “smart” biomaterials that are able to replicate the complex structure of tissue and chemically communicate with cells cultured within them has thus far proved to be a challenging task. To this end we recently developed magnetic vesicle gels, a new type of biomaterial composed of adhesive lipid-doped thermally sensitive vesicles (TSVs) crosslinked with magnetic nanoparticles. The resulting magnetic nanoparticle-vesicle assemblies are then embedded in a biocompatible hydrogel to give a magnetic vesicle gel.\textsuperscript{1} The magnetic functionality in these materials allows non-invasive magnetospatial control of vesicle-nanoparticle assemblies\textsuperscript{2} in the gel and facilitates magnetic release of the vesicle contents into the surrounding volume. The application of an alternating magnetic field (AMF) releases bioactive compounds stored in the vesicles, which then diffuse through the gel and trigger cellular responses.

A key part in the design of these materials is the hydrogel scaffold that immobilizes the magnetic nanoparticle-vesicle assemblies and provides the local environment that supports cell growth. Previously we used a calcium alginate scaffold, as we found this material held the assemblies in place without the gel fibrils disrupting the TSV membranes.\textsuperscript{1} Alginate gels are also easily formed and
Appendix

manipulated at physiological temperatures, which was hoped to allow these magnetic vesicle gels to be applied to biological systems. Nonetheless, calcium alginate is a poor scaffold for the proliferation of several types of cell. To improve the versatility of these vesicle gels for cell culture applications, several other gel scaffolds were tested for compatibility with TSVs and several cell lines.

**EXPERIMENTAL DETAILS**

\(N\)-(Biotinoyl)dopamine was synthesized by a modification of literature procedures.\(^5\)

Magnetic nanoparticles (MNPs) were formed as previously detailed\(^2\) from iron(II) chloride tetrahydrate and iron (III) chloride hexahydrate. These iron salts were dissolved in deoxygenated water then added dropwise to NaOH solution (1 M) under nitrogen with vigorous stirring over 30 minutes. The particles were then magnetically sedimented using a N48 5350 G neodymium iron boron magnet and washed to remove any nonmagnetic material. The magnetic nanoparticles (12.5 mg) were suspended in methanol (3 mL) under nitrogen, then mixed with \(N\)-(biotinoyl)dopamine (3.5 mg, \(9.2 \times 10^{-6}\) mol) and sonicated for 4 hours. The coated particles were magnetically sedimented using an NdBFe magnet and washed with methanol repeatedly, using a magnet to sediment the particles between washes, to give coated MNPs as a fine brown powder.

Thermally sensitive vesicles (TSVs) were composed of 4:1 mol/mol dipalmitoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidylcholine (DMPC), with 1 % mol/mol triethylammonium (\(N\)-(biotinoyl)-1,2-dihexadecanoylsn-glycero-3-phosphoethanolamine (Bt-DHPE, from Sigma) added if the TSVs were to be crosslinked with magnetic nanoparticles. The lipids (DMPC, 1.27 mg; DPPC, 12.55mg; Bt-DHPE, 200μL from 1 mM stock solution in CHCl\(_3\) if required) were dissolved in chloroform (1 mL) and the solvent removed under reduced pressure to give a lipid thin film on the inside of the round-bottomed flask. The lipid film was hydrated in 0.05M 5/6-carboxyfluorescein (5/6-CF) in MOPS buffer at pH 7.4 (1 mL) then extruded through a single 800 nm polycarbonate membrane (19 ×) at > 40 °C to give ~800 nm diameter biotin-tagged TSVs.
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The magnetic nanoparticle-vesicle assemblies were formed immediately prior to use by mixing biotin coated nanoparticles and the biotin-tagged TSVs with avidin in a 2:1 biotin:avidin ratio, and allowed to aggregate for 60 min. Magnetic sedimentation was used to remove unencapsulated 5/6-CF and non-aggregated vesicles. The vesicle suspensions were placed upon a 5 kG magnet until a compact vesicle plug had formed at the bottom of the vial and the supernatant solution was visually free of turbidity. As much of the supernatant was removed as possible without disturbing the TSV plug (typically 60% of the volume), and replaced with an equal volume of the appropriate buffer solution. Brief vortex mixing regenerated the vesicle suspension. This procedure was repeated at least 6 times and until the concentration of unencapsulated 5/6-CF was < 0.1% of the initial concentration.

The cells were seeded in gel scaffolds at a cell count of 5 cells/μL for 3T3 fibroblasts and 10 cells/μL for chondrocytes; 3T3 fibroblasts proliferate much faster than chondrocytes and were therefore seeded at a lower cell count, generally 20,000 vs. 40,000. The gel matrix was then added to the cell suspension in thin films of gel (0.500 mL per well in 24 well plates and 0.800 mL per well in 12 well plate). The gel scaffolds were then suspended in fresh DMEM media to allow transfer of cell nutrients and waste. The metabolic activity and DNA count of the cell lines were measured to study the reaction of the cells to their surroundings.

Alginate gels were formed by stirring alginic acid (2 % wt/vol) in PBS for 60 minutes at room temperature. The gel was formed by infusion of CaCl₂ (0.1 M) through a 800 nm polycarbonate membrane. If required, fibronectin solution (1 mg/mL) was added at 1 % vol/vol. The gels were then sterilized by autoclaving at 120°C for 30 minutes. Chitosan was added to well plate, then cured using PBS whilst adding cell solution into the gel. Chitosan:PEI gels were formed by suspending cells in PEI solution (30 % wt/vol in PBS, pH 11) and then adding this solution to chitosan (2 % wt/vol in aqueous acetic acid, pH 3); the gel formed upon mixing. Cells were suspended in gelatin solution (1 % wt/vol), then cooled to room temperature for gelation.

DISCUSSION

Testing new hydrogels for compatibility with DPPC:DMPC TSVs

In addition to calcium alginate, four other types of gel that have been used for cell culture were tested for compatibility with TSVs. Mixing chitosan and polyethyleneimine (PEI) in a 50:50 ratio under physiological conditions will lead to stable gels that have been shown to support cell proliferation. This procedure does not require external stimuli such as heat, sonication or pH changes, which would disrupt the membranes of the TSVs in the gel. Similarly chitosan itself, although it requires a pH change to gel, allows sufficient time to add the TSVs to the mixture after buffer addition (phosphate buffer, pH 7.4) before the hydrogel gel forms.
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Gelatin, a widely used hydrogel scaffold, was also tested. Although heating is required for gelation, after heating the gelatin remains fluid at room temperature for 5 minutes, allowing TSVs to be added at 25 °C before full gelation occurred. Finally, as alginate has already been established as a good scaffold for magnetic nanoparticle-vesicle assemblies, this material was modified to increase cell attachment and proliferation by doping the alginate matrix with fibronectin. Fibronectin, a high molecular weight glycoprotein, is found in the extracellular matrix and can significantly enhance cell adhesion, also playing a role in cell differentiation and migration.4

To test for vesicle membrane disruption during the gelation of these mixtures, TSVs with encapsulated carboxyfluorescein were mixed with the hydrogelator in buffer. The solution was allowed to gel and the change in fluorescence was monitored over time under cell culture conditions (37 °C in a 5 % CO₂ atmosphere), with an increase in fluorescence indicating that the gelation process had disrupted the vesicle membrane.

![Figure 1](image)

**Figure 1:** (a) Leakage of 5/6-carboxyfluorescein from thermally sensitive vesicles (TSVs) embedded in different gel matrices at 37 °C (♦) Alginate:fibronectin (○) Chitosan (●) Chitosan:PEI (∗) Gelatin (■) Alginate. (b) Magnetic release of 5/6-carboxyfluorescein encapsulated in magnetic nanoparticle-vesicle assemblies (in suspension, MOPs buffer pH 7.4), after exposure to a 2 minute pulse of 392 kHz alternating magnetic field (AMF) at 5 minutes.

At 37 °C there was significant leakage of the TSV contents in all gel mixtures over a 6 hour period (Fig. 1 (a)), which can be ascribed to the proximity of the $T_m$ of this TSV composition to the temperature required for cell culture. This is despite the 1-2 degree increase in $T_m$ anticipated from previous studies of these 4:1 DPPC:DMPC vesicles in alginate gels.1 Chitosan and gelatin were the least suitable gel matrices as gelation of these mixtures caused 100% release of the encapsulated
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dye by the first time point (1 hour). The high rate of leakage from the TSVs in chitosan may be due to incomplete neutralization of the gel, with the low pH causing extensive membrane disruption. The gelatin gels also showed poor stability under these conditions. The other gel matrices showed little difference in the rate of dye release. Interestingly the undoped alginate gel showed slightly increased rate of leakage compared to alginate doped with 1% fibronectin, which may indicate some bilayer-protein interactions.

Clearly the conditions used to form chitosan and gelatin gels are incompatible with the inclusion of TSVs in these gels, but this 4:1 DPPC:DMPC TSV composition also appears to have a \( T_m \) that is too low to be useful for cell culture in magnetic vesicle gels. Despite the relatively rapid rate of contents release from this TSV composition under magnetic stimulation (release of 5/6-CF is shown in Fig 1 (b)), the response of the cells to the released stimuli is likely to take hours to days, making the observed background rate of release too high in all hydrogels.

Testing new hydrogels for compatibility with 3T3 fibroblasts and chondrocytes

The three hydrogel scaffolds that were shown to give the slowest release of encapsulated contents at 37 °C (alginate, alginate-fibronectin, chitosan:PEI) were tested for their ability to support the proliferation of two different cell lines; 3T3 fibroblasts and chondrocytes.

The 3T3 cell line is a robust cell line of Swiss mouse fibroblasts with a rapid proliferation rate, making this an excellent cell line to trial our methodology. Furthermore the effect of the gel composition on cell proliferation could clearly be analyzed using the live/dead assay.

![Figure 2](image)

**Figure 2:** (a) Metabolic activity of 3T3 fibroblasts on gel scaffolds as determined by the alamar blue assay; (b) Proliferation of 3T3 fibroblasts on gel scaffolds as determined by the DNA assay; (■) Alginate/fibronectin (●) Alginate (♦) Chitosan/PEI.
Cell counts on the gel samples show that doping the alginate gel with fibronectin significantly increases metabolic activity and the rate of cell proliferation, while the undoped alginate scaffold supports cell proliferation much better than chitosan-PEI. All these gel scaffolds were however much less effective than tissue culture plastic at supporting cell growth, with even the alginate-fibronectin mixture giving less than 10% of the cell count of this control. The chitosan:PEI gels also presented several experimental problems, as they were difficult to seed, had poorly reproducible gel stiffness and interfered with assays using pH sensitive dyes like alamar blue. The morphology of the cells studied by live/dead assays shows that when fibronectin is present the cells attach to the surrounding scaffold and spread, while in alginate only cells remain rounded with no attachment points.

When chondrocytes were cultured on these same three scaffolds (alginate, alginate:fibronectin and chitosan:PEI), the alamar blue metabolic activity assay showed that the activity of these cells generally increased over time as the cells proliferated (Figure 3 (a)). Little difference in chondrocyte activity was observed between the undoped alginate and the fibronectin doped alginate scaffold, possibly because chondrocytes can to some extent maintain themselves in a rounded form and the presence of RGD attachment points in the matrix is not a crucial as for fibroblasts. In addition, the external addition of calcium(II) to either chondrocytes or fibroblasts revealed that chondrocytes appeared to have a higher tolerance for calcium(II) in the surrounding matrix. In comparison the DNA assay of cell number shows that the undoped alginate scaffold gave the best cell proliferation (Figure 3 (b)), followed by the alginate:fibronectin mixture. Chitosan:PEI scaffolds did initially support faster proliferation of chondrocytes than the alginate based gels, but this sharply declined after a few media changes. In comparison few cells survived exposure to pure chitosan gel; separate tests on pure chitosan scaffolds showed no cell proliferation until 10 days had passed.
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Figure 3: (a) Metabolic activity of chondrocytes on gel scaffolds as determined by the alamar blue assay; (b) Proliferation of chondrocytes on gel scaffolds as determined by DNA assay; (■) Alginate/fibronectin (●) Alginate (♦) Chitosan/PEI.

CONCLUSIONS

Of the five hydrogels tested for compatibility with thermally sensitive vesicles (TSVs), 3T3 fibroblasts and chondrocytes, alginate-based gels showed the greatest versatility for the development of new types of magnetic vesicle gels. Both gelatin and chitosan gels were found to be incompatible with TSVs and caused extensive membrane disruption, while further tests on chitosan gels also showed that they did not support cell growth. Mixing chitosan with PEI produced gels that were more cell-compatible, but the materials properties of these mixed gels were inconsistent and the gels proved to be poorer supports for cell growth than alginate gels. The chief drawback of undoped calcium alginate gels was the lack of cell attachment points in the matrix. This was a less significant problem for chondrocytes than for fibroblasts, but the addition of fibronectin at 1 % gave significant improvements in 3T3 fibroblast proliferation without significantly changing TSV compatibility. Nonetheless studies on dye leakage from TSVs in alginate gels at 37 °C revealed that the 4:1 DPPC:DMPC composition in the magnetic vesicle gels that were developed previously would be unsuitable for cell culture. Future development of these “smart” biomaterials will require TSV compositions with higher melting temperatures. These high $T_m$ TSVs will be mixed with calcium alginate gels and cell viability in the resulting magnetic vesicle gels assessed under physiological conditions.

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REFERENCES

Appendix