Preparation and characterization techniques for nano-structured materials

A thesis submitted to the University of Manchester for the degree of

PhD

in the Faculty of Medical and Human Sciences

2011

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Abstract

The first part of the project focused on the optimization of processes for the preparation of enzyme-containing silicagel nanoparticles and for their coating and stabilization with a polycationic layer. A procedure for coating surfaces with polymer layers was established. Atom transfer radical polymerization was used for the synthesis of a cationic macroinitiator adsorbed on the anionic surface of near-monodisperse silica nanoparticles, used as model for enzyme-containing silicagel nanoparticles. The latter were easily purified via gel filtration, while enzymatic activity was substantially retained during both macroinitiator adsorption and gel filtration. The subsequent growth of water soluble poly (glycerol monomethacrylate) (pGMMA) via ATRP onto coated enzyme-containing silicagel nanoparticles was achieved in a living fashion and with a substantial retention of the activity of encapsulated enzymes. The decoration of the surface with hydrophilic and protein-repellent polymers can provide “stealth” properties to the supported enzymes, which can be eventually functionalized to obtain more sophisticated biologically responsive nanoparticles.

In the second part of the project characterization of nano-structured materials at sub-nanometer resolution was achieved by Atomic Force Spectroscopy (AFM) to probe simultaneously the structure and specific chemical and physical parameters of the system. At the same time, the force-deformation behavior of nano-structured materials subjected to concentrated loads (nanoindentation) yield detailed information and insight about their local mechanical and adhesion properties. In particular, we have focused on the characterization of nanoparticles, surface layers and self-assembled fibrillar materials, combining imaging with a local mechanical (Young’s modulus) and physical (adhesion force and surface energy) analysis of the materials.
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Dedication

To my Family and Friends
Acknowledgements

I take this opportunity to express my deepest appreciation and gratitude to Prof. Nicola Tirelli for offering me this PhD-Position and guiding me through this journey. I am profoundly thankful for his outstanding engagement he showed in every discussions and explanations, for the passion of research he transmitted to me, for the encouraging attitude he always promoted. Nicola has also been a person of reference for me during all my stay in the United Kingdom, and for that I am absolutely very grateful.

This work would not have been possible without the help of many individuals. I would like to particularly thank Dr. Francesco Cellesi for his valuable advices and support, for flanking me in my research activity, and all the other people in the group of the Laboratory of Polymers and Biomaterials. I would like to thanks all my friends in Manchester with whom I shared special moments and showed infinite patience and moral support. Last but not least, my thanks go to my family and to my Italian friends, for their love and their support in any occasion.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ATRP</td>
<td>Atom Transfer Radical Polymerization</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force microscopy</td>
</tr>
<tr>
<td>ATR-IR</td>
<td>Attenuated Total Reflectance Infra Red (spectroscopy)</td>
</tr>
<tr>
<td>Bpy</td>
<td>2,2’-bipyridyl</td>
</tr>
<tr>
<td>BriBuBr</td>
<td>α-bromoisobutyryl bromide</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>CuBr</td>
<td>Copper(I)bromide</td>
</tr>
<tr>
<td>CuCl</td>
<td>Copper(I)chloride</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>Copper(II)chloride</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of Polymerization</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMAEMA</td>
<td>2-(dimethylamino)ethyl methacrylate</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>EB/B</td>
<td>Ethyl α-bromoisobutyrate</td>
</tr>
<tr>
<td>GG</td>
<td>Fmoc-diglycine</td>
</tr>
<tr>
<td>FF</td>
<td>Fmoc-diphenylalanine</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
</tr>
<tr>
<td>GMMA</td>
<td>Glycerol monomethacrylate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>NIPAAm</td>
<td>N-isopropylacrylamide</td>
</tr>
<tr>
<td>G''</td>
<td>Loss modulus</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>CH3I</td>
<td>Methyl iodide</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
<td>η</td>
<td>Newtonian viscosity</td>
</tr>
<tr>
<td>$M_n$</td>
<td>Number Average Molecular Weight</td>
</tr>
<tr>
<td>TPP</td>
<td>Pentasodium Triphosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>$M_w / M_n$</td>
<td>Polydispersity Index</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>Proton Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SiO$_2$</td>
<td>Silica</td>
</tr>
<tr>
<td>G</td>
<td>Shear modulus</td>
</tr>
<tr>
<td>G'</td>
<td>Shear storage</td>
</tr>
<tr>
<td>$\eta_0$</td>
<td>Steady state viscosity</td>
</tr>
<tr>
<td>TEOS</td>
<td>Tetraethoxysilane</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric Analysis</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>UV-VIS</td>
<td>Ultra Violet-Visible (spectroscopy)</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>Water</td>
</tr>
<tr>
<td>$M_w$</td>
<td>Weight Average Molecular Weight</td>
</tr>
<tr>
<td>% wt</td>
<td>Weight Percent</td>
</tr>
<tr>
<td>E</td>
<td>Young Modulus</td>
</tr>
<tr>
<td>kDa</td>
<td>10$^{-3}$ Dalton</td>
</tr>
<tr>
<td>mg</td>
<td>10$^{-3}$ gram</td>
</tr>
<tr>
<td>kHz</td>
<td>10$^{-3}$ Hertz</td>
</tr>
<tr>
<td>μl</td>
<td>10$^{-6}$ liter</td>
</tr>
</tbody>
</table>
mL $10^{-3}$ liter
μm $10^{-6}$ meter
nm $10^{-9}$ meter
M $mol \cdot liter^{-1}$
mmol $10^{-3}$ mol
mV $10^{-3}$ Volt
nN $10^{-9}$ Newton
kPa $10^3$ Pascal
1 Introduction

1.1 Summary

Nanotechnology has achieved a number of breakthroughs in bioengineering, molecular biology, diagnostics, and therapeutics. Among the others, a major advance is the development of a (bio)functional nanosystems through the incorporation, adsorption, or covalent coupling of synthetic polymers, carbohydrates, peptides, proteins, nucleic acids, and polysaccharides to the surface of nanoparticles. Functionalization confers a wide array of interesting properties such as “stealth” characteristics, bioadhesion and targeting, while it may also prevent aggregation of nanoparticles, impart biostability and solubility, reduces toxicity, and provides site-specific delivery. This makes such nanosystems an intelligent tool for diagnostics, prognostics, and controlled and sustained delivery of therapeutic agents to specific targets (tissue, cell, and intracellular) for e.g. the treatment of cancer, genetic diseases and other ailments.

Injectable, surface-decorated and long-circulating (“stealth”) nanoparticles are thus specifically advantageous for systemic, parenteral delivery applications, because their small size helps to prevent embolization, while their surface chemistry their uptake by the mononuclear phagocyte system (MPS). Their size also allows for passive targeting effects, such the Enhanced Permeation and Retention (EPR) effect in solid tumours.

The first aim of this project has focused on the development of enzyme-containing nanoparticles, with the aim to prepare long-circulating nanoparticles capable of performing reactions that are not accessible to mammalian enzymes, without however triggering immune reactions. A bacterial enzyme can therefore be protected by degradation, made non-immunogenic and possibly targeted through the use of a “stealth” nanoparticle host, while being still active and therefore able to produce chemical conversions that are not possible for human enzymes. In this way an enzymatically activated pro-drug could be converted only at the location of nanoparticle accumulation, e.g. a tumoral site.

This part of the project has essentially hinged on the use of modern polymer chemistry
techniques (ATRP) for the design of surface-functionalized enzyme-containing nanoparticles. However, for a better understanding of such nano-structured materials and of their interactions with biological systems at the nanoscale, high-resolution imaging and analysis tools are required that can provide nano-resolution in an aqueous environment and under physiological conditions. Atomic force microscopy (AFM) is such a tool. With its ability to observe, manipulate and explore the functional components of the nano-structured material at sub-nanometer resolution, it has produced a wealth of new opportunities in nanotechnology. The use of the AFM probe as a ‘lab-on-a-tip’ enables us to probe simultaneously the structure and specific chemical, physical and biological parameters of the system. At the same time, the force-deformation behavior of nano-structured materials subjected to concentrated loads can yield detailed information and insight about their local mechanical and adhesion properties. The second aim of the project was thus to employ AFM for the imaging of biomaterial surfaces under physiological conditions. In particular, we have focused on the characterization of nanoparticles, surface layers and self-assembled fibrillar materials, combining imaging with a local mechanical (Young’s modulus) and physical (adhesion force and surface energy) analysis of the materials.

1.2 Preamble

The development of new materials for the technological demands of the 21st century is a major goal of materials science. The ‘classical’ materials have often reached their limits, especially in areas such as microelectronics, optics, sensor technology, catalysis, ceramics, etc. Therefore, new materials with tunable properties are needed. A class of materials which could meet this requirement are hybrid materials, especially those in which inorganic and organic components are combined. These systems merge, to some extent, the properties of the two components on a molecular scale and therefore allow a deliberate tailoring of properties between purely inorganic and purely organic materials. A special challenge of increasing importance is to tailor not only the
composition of such materials, but also their structures especially in the nanometer range.

Recent advances in nanotechnology have opened up a wide range of highly sophisticated and promising biorelated applications for smaller and smarter functionalized nanoparticles \cite{1, 2}. Nanoparticles with tailored properties and flexible surface functionalization can be used to generate a multifunctional platform for various biomedical applications \cite{3}. Surface properties and compositions of the nanoparticles are key issues to address for application in complex biosystems. Ideally, surface modification of nanoparticles should provide good colloidal stability, biocompatibility, and specific biorecognition of the particle surface \cite{4, 5}.

A relevant example is offered by inorganic nanoparticles (metal and above all metal oxide nanoparticles), which have been widely used in drug delivery, biodetection, and medical imaging due to their optical and electrochemical properties. In most cases, hydrophilic, protein-repellent polymers are used to coat the surface and functionalize the metal nanoparticles, serving as a protective shield to prevent nonspecific interactions with biological systems and improve efficacy and stability of these nanosystems: for instance, core–shell structure magnetic particles with polymer brushes with a large amount of active sites on the shell brushes, can combine the advantages of easy separation from the reaction medium and high loading capacity providing carriers for enzyme immobilization, biosensors and industrial biocatalysts \cite{6}.

In this project we have tackled the generic target of producing complex nanoparticles characterized by a) an inorganic core possibly bearing encapsulated active principle, b) a hydrophilic shell composed of hydrophilic polymers and produced from the inorganic core through controlled/living polymerization which would allow controlling the shell thickness.

We will specifically review the features of controlled/living radical polymerization and its application to the functionalization of colloidal objects.
1.3 Controlled (living) polymerization mechanisms

1.3.1 General definitions

The mechanism of chain-growth polymerisation, employed for instance in free radical, anionic and cationic polymerisation, presents three distinguished elementary steps (Erreur ! Source du renvoi introuvable.): a) initiation, b) propagation and, c) termination (e.g. through coupling, disproportionation, spontaneous deactivation or chain transfer).

Figure 1-1. Common polymerisation steps in a free radical polymerisation; first radical ($R^\ast$) is generated by decomposition of the initiator and the first monomer is attacked (a), the polymerisation then propagates by addition of other monomers (b) until termination reactions (c) take place (for free radical polymerisation this may happen through coupling or disproportionation; please note that these are not the only possible termination reactions).

Spontaneous and uncontrolled termination reactions often hinder the preparation of polymers with narrow molecular weight distribution and precisely controlled architecture (poor control over terminal groups).

In a “living” polymerisation, on the contrary, the propagating specie at a terminus of the chain is never de-activated, also after all monomer has been polymerised; therefore, when fresh monomer is added, polymerisation can resume.

Among the earliest examples of living polymerisation, it is possible to go back as early as 1936, when Ziegler [7] proposed the anionic polymerisation of styrene and butadiene (initiated by alkyl lithium) to possibly occur without chain transfer and termination; it is
nevertheless only in 1956 that Szwarc \[8\] first demonstrated and reported on the living character of anionic polymerisation (styrene initiated from sodium naphthalene in THF), synthesising particularly well-defined block copolymers through the addition of isoprene monomers after depletion of styrene.

In a general case, it is accepted that living polymerisations are chain-growth reactions that propagate: a) without appreciable termination reaction and b) possibly without chain transfer (or, better, irreversible chain transfer) \[9\]. Provided that initiation is a quantitative reaction and is at least as fast as the propagation reaction, so that the number of kinetic chain carriers is essentially constant throughout the polymerisation, the theoretical molecular weight (or the degree of polymerisation, DP) linearly decreases with increasing initiator concentration (eq. 1-1 and Figure 1-2, trace A). At the same time, the MW distribution assumes a Poisson-type shape and the polydispersity decreases with increasing degree of polymerisation (Figure 1-2, dotted line A) \[10\],\[11\].

Most commonly, the final molecular weight distribution is expected to have a polydispersity index comprised in the range \(1.0 < \frac{M_n}{M_w} < 1.5\) \[12\].

\[
DP = \left( \frac{[M]_0}{[Initiator]_0} \right) \times conversion \quad \text{Eq. 1-1}
\]

\[\text{with} \quad 0 < conversion < 1\]
The most important benefit of controlled/“living” polymerizations is that they allow preparation of new macromolecules with varying compositions (see Erreur ! Source du renvoi introuvable.3) (homopolymers, random, periodic, block, graft and gradient copolymers), novel topologies (linear, star, comb, (hyper) branched, networks, etc.) and functionalities placed at different parts of macromolecules or various combinations of
these; materials with vastly different properties can be then easily created from the same set of monomers.

Since the first description of living polymerisations, for long the anionic mechanism has been the mechanism of choice for the preparation of the polymers with very narrow molecular weight distribution.

The last 15 years, on the other hand, have seen the development of a few mechanisms of controlled/living radical polymerisation, driven by the industrial relevance of radical polymerizations and the robustness (e.g. tolerance to water) of this type of mechanisms. Notably, at the dawn of the development of controlled radical mechanisms (1995), polymers obtained through free radical polymerisation accounted approximately half of the total production of polymers in the United States \(^{13}\). The major problem of these
industrially important processes is the fast and irreversible termination of the growing chains through coupling and disproportionation reactions. This problem has been circumvented through a general approach of establishing a rapid dynamic equilibrium between a small amount of growing radicals and a large majority of “dormant” species. The dormant chains may be alkyl halides, as in atom transfer radical polymerisation (ATRP) \[4, 14, 15\] or degenerative transfer (DT), thioesters, as in reversible addition fragmentation chain transfer processes (RAFT) \[16\], alkoxyamines, as in nitroxide mediated polymerisation (NMP) \[17\] or stable free radical polymerisation (SFRP); in this manner, a small concentration of radicals can be employed to propagate a large number of chains. For this equilibrium to be effective in controlling a radical polymerization, there are two necessary conditions: first, the equilibrium between dormant and active (free radical) species must lie strongly to the side of the dormant species to assure that the overall concentration of radicals will remain very low and that the rate of irreversible termination will be negligible relative to the apparent rate of polymerization; second, the rate of exchange between dormant and active species must be faster than the rate of propagation to assure that all polymer chains have an equal probability of adding monomer. Moreover the irreversible termination is only minimized in these polymerizations and not excluded from the mechanism. Therefore, these polymerizations do not meet the strict definition of a living polymerization and are more properly termed controlled/living polymerizations to reflect the uncertainty regarding the contribution of unavoidable irreversible termination. However, above some molecular weight value specific to the polymerization of each monomer, all controlled/living radical polymerizations can no longer be considered strictly controlled, because slower termination, transfer and other side reactions become significant. In the next sections we will focus on the mechanism and properties of atom transfer radical polymerization, due to the relevance of these mechanisms for the polymerization reactions described in this thesis.
1.3.2 Atom transfer radical polymerization (ATRP)

Atom transfer radical addition (ATRA) or more generally the Kharasch addition reaction is so named because it employs atom transfer from an organic halide to a transition-metal complex to generate the reacting radicals, followed by back transfer from the transition metal to a product radical to form the final product. In ATRA (scheme 1) a metal catalyst, usually a complex of a copper (I) halide and 2,2'-bipyridyl [18], [19], [20], [21], [22], (although Ni [23], Pd [24], Ru [25], [26], Fe [27] and other metals [28] have been used as well) undergoes a one-electron oxidation with concomitant abstraction of a halogen atom from a substrate.

This reaction generates an organic radical and a copper (II) complex. One requirement for the reaction to occur is that substituent must be present on the organic halide that will stabilize the resultant radical which can add to an unsaturated compound in an inter- or intramolecular fashion, or it can abstract the halogen atom from the copper (II) complex and revert back to the original dormant organic halide species. The copper (I) complex is reformed, completing the catalytic cycle. The radical may also react with another radical, but since the concentration of propagating radicals is very small, the contribution from termination reactions to the products formed is minimal. The substrates for this reaction are typically chosen in order that, if addition occurs, the newly formed radical would be much less stabilized than the initial radical and will essentially react irreversibly with the copper (II) complex to form an inactive alkyl halide product. Therefore, in ATRA usually only one addition step occurs.

Atom transfer radical addition can be extended to atom transfer radical polymerization (ATRP) if the conditions can be modified such that more than one addition step is possible (scheme 1-1), so if the radical species before and after addition of the unsaturated substrate possess comparable stabilization, then the activation - addition - deactivation cycle will repeat until all of the unsaturated substrate present is consumed [6]. ATRP was first reported in 1994/1995 by Sawamoto and co-workers [29], [30], [31] and Matyjaszewski and co-workers [8], [9], [32].
1.3.2.1 ATRP mechanism and kinetics

An ATRP system is composed of an initiator, a metal halide complexed with some ligand(s) and a monomer.

The mechanism consists of initiation and propagation processes that are phenomenologically related: these sequences are comprised of an atom transfer equilibrium and an addition of the intermediate radical to a monomer.

Scheme 1-1. General mechanism for ATRA (A) and ATRP (B) $L_n T = $ metal complex at a certain valence state $z$, $X =$ halogen (Cl or Br), and $M =$ Monomer.
During initiation, the radicals species are generated by a reversible redox process catalyzed by a transition metal complex that extracts the halogen atom X from the initiator RX and generates a radical initiator R\(^*\) which is added to the monomer; the complex in its higher oxidation state promotes an inverse redox reaction in which the radical is deactivated producing the dormant species and regenerating the complex in its initial oxidation state; the polymeric chains thus propagate adding the monomer to the growing chain end.

To achieve polymers with low polydispersity it is necessary that the reversible deactivation of the growing chain occurs very fast, therefore \(k_d > k_a\) (scheme 1-1). An equilibrium shifted towards the dormant species allows to have a low radicals concentration and thus to minimize the termination events by coupling.

The apparent constant rate of the initiation process is \(k_i^{\text{app}} = k_i K_0\), where \(k_i\) is the absolute constant rate of the monomer addition stage to the first formed radical and \(K_0 = k_a^0/k_d^0\) is the equilibrium constant of the atom transfer reaction in the initiation process; therefore \(k_a^0\) has to be higher than \(k_d^0\) in order to promote the first radical formation; if \(k_a^0\) is too high and \(k_d^0 \approx 0\), the initiation is too quick and it is thus produced an excessive number of radicals giving a significant irreversible termination, reducing the initiator effectiveness and decreasing the polymerization rate; \(k_i^{\text{app}}\) has to be comparable to the apparent propagation constant \(k_p^{\text{app}} = k_p K_{eq}\) whereas \(k_p\) is a constant rate of the propagation state and \(K_{eq}\) is the equilibrium constant of the atom transfer reaction in the propagation process; if the \(k_i^{\text{app}} \ll k_p^{\text{app}}\) polymers with higher molecular weights than the theoretical ones and with higher polydispersities are yielded.

The termination occurs mainly at the beginning of the polymerization producing a terminated chain percentage not higher than 5% [6]. As previously mentioned, in ATRP and other controlled radical polymerizations, termination always occurs but it becomes insignificant due to the persistent radical effect [33], [34]; the stable radical is often called the persistent radical and its suppression of bimolecular termination between living polymers is called the persistent radical effect (PRE).
In case of a fast equilibrium approximation (necessary condition for observing low polydispersities) and omitting the termination step the rate law for this mechanism can be derived:

\[ R_p = k_{app} [M] = k_p [P^•] [M] = k_p K_{eq} [RX] [Cu^I] [M] / [Cu^{II}X] \quad \text{Eq. 1-2} \]

where \([M]\) is the monomer concentration, \([P^•]\) is the radical species concentration, \(k_p\) is the propagation constant, \(k_{app}\) is the apparent rate constant, \([RX]\) is the initiator concentration, \([Cu^I]\) is the copper (I) concentration and \([Cu^{II}X]\) is the copper (II) complex concentration and 

\[ K_{eq} = k_a / k_d = [P^•] [Cu^{II}X] / [Cu^I] [PX]. \]

In a controlled polymerization the initiation process is very fast, therefore the active species concentration \([P^•]\) is equal to the product of the initial initiator concentration \([RX]_0\) times the ratio between Cu(I) and Cu(II) at the equilibrium, and it remains constant along the process in the absence of termination reactions; therefore from the eq. 1-2 it can be deduced a first order kinetic equation in respect to the monomer concentration:

\[ \ln[M]_0/[M] = k_p K_{eq}[M][P^•]t = k_{obs} \cdot t \quad \text{Eq. 1-3} \]

Plotting the graph \(\ln[M_0]/[M]\) versus time it is obtained a linear first order kinetic diagram; in the presence of termination the Cu (II) concentration would increase, leading to a curved kinetic diagram.

As in living polymerizations, in a controlled ATRP reaction the average molecular weight increases in a predictable and linear fashion with the conversion and it is controlled by the initial stoichiometry of the reagents, allowing the achievement of polymers with predetermined final molecular weight (eq.1-1); the polidispersity decreases with the monomer conversion (p) as predicted from the equation:
In absence of termination and transfer reaction, the polydispersity index depends upon the initial initiator concentration \([RX]_0\), the deactivating species concentration \([\text{Cu(II)}]\), the propagation constant \(k_p\), the deactivation constant \(k_d\) and the conversion.

The higher polydispersities are thus presented by polymers with a significant propagation constant and by low molecular weight polymers, because they are achieved using an initial consistent amount of initiator \([RX]_0\).

However equation 1-4 is true only if the initiator is completely consumed and the degree of polymerization is quite high (\(DP > 200\)), otherwise the Poisson factor \(\left(\frac{M_w}{M_n} = 1 + \frac{1}{DP}\right)\) has to be taken into account.

1.3.2.2. **Monomer**

The choice of monomer determines also the choice of all the other polymerization parameters; each monomer possesses an intrinsic radical propagation rate, so the concentration of propagating radicals and the rate of radical deactivation may need to be adjusted to maintain polymerization control.

In the absence of any side reactions other than radical termination by coupling or disproportionation, the magnitude of the equilibrium constant \((K_{eq})\) determines the polymerization rate. ATRP will not occur or occur very slowly if the equilibrium constant is too small. In contrast, a too large equilibrium constant will lead to a large amount of termination events because of a high radical concentration; this will be accompanied by a large amount of deactivating higher oxidation state metal complex which will shift the equilibrium toward dormant species and may result in the slower polymerization \([35]\); typical monomers include styrenes, acrylates, (meth)acrylates, (meth)acrylamides, and
several other relatively reactive monomers, such as vinylpyridine and acrylonitrile, which contain substituents that can stabilize the propagating radicals. The polymerization of acrylamides under typical ATRP conditions displayed a much lower ATRP equilibrium constant than the acrylates or styrenes \cite{36} probably due to the inactivation of the catalyst by complexation of copper by the forming polymer and displacement of the terminal halogen atom by the amide group: the competition between the halogen atom and the amide group of the polymer in the catalyst complexation would lead thus to a lower catalyst activity. The easier polymerizability of (meth)acrylates for the ATRP reaction is due to the easier activation of the dormant species and thus higher values of the ATRP equilibrium constants \cite{10}.

1.3.2.3. Initiator

The main role of the initiator is to determine the number of growing polymer chains although sometimes function can be equally important; if initiation is fast and transfer and irreversible termination events negligible, then the number of growing chains is constant and equal to the initial initiator concentration. For the polymerization of each monomer, the corresponding initiator end group will possess its own unique redox potential. Therefore, in combination with the same metal catalyst, each end group will exhibit different atom transfer equilibrium constant, deactivation rate constant and corresponding concentration of propagating radicals. The initiator usually, but not always, should have a structure homologous to the corresponding end group of the propagating chain; in the case of initiators that are not structurally related to the dormant polymer chain end, it is better to use organic halides that form less reactive radicals with higher efficiency than the dormant polymer chain ends; moreover, the halogen atom in the initiator and that in the metal complex can be identical but this is not necessary; however the halogen atom, X, must rapidly and selectively migrate between the growing chain and the transition metal complex.
In general, any alkyl halide with activating substituents on the $\alpha$-carbon, such as aryl, carbonyl and allyl groups, can potentially be used as ATRP initiators; polyhalogenated compounds (CCl$_4$ and CHCl$_3$) and compounds with a weak R-X bond, such as N-X, S-X and O-X can also be used as ATRP initiators; however chloride and bromide initiators are the ones that assure the better control of molecular weight because they allow achieving a rapid and selective transfer from the growing chain to the complex $^6$.

1.3.2.4. Catalyst: transition metals and ligands

The catalyst has a key role in ATRP since it determines the position of the atom transfer equilibrium and the dynamics of exchange between the dormant and active species. There are several requirements for an effective ATRP catalyst: first, the metal complex must have an accessible one-electron redox couple to promote atom transfer and its metal center should have reasonable affinity toward a halogen. Secondly, the coordination number of the metal center must increase by one in order to accommodate a new ligand. In most systems the lower oxidation state of the metal is presumed to be tetracoordinate and the higher oxidation state is presumed to be pentacoordinate. Moreover a good ATRP catalyst must show selectivity for atom transfer and therefore possess a low affinity for alkyl radicals and the hydrogen atoms on alkyl groups.

Therefore an efficient catalyst must permit a fast and quantitative initiation so that all the chains start to grow simultaneously; secondly, it has to shift neatly the equilibrium towards the dormant species in order to have a low radical concentration and minimize the termination reactions. Finally it has to allow a quick deactivation of the active radicals by the halogen transfer to yield low polydispersities.

The choice of ligand greatly influences the effectiveness of the catalyst: the main roles of the ligand in ATRP are to solubilise the transition-metal salt in the organic media, tuning the Cu catalyst activity, and to adjust the redox potential of the metal center for appropriate reactivity and dynamics for the atom transfer $^{37}$. Since the catalyst is not bound to the growing chain, ATRP should behave in a similar manner whether or not
the catalyst is highly soluble in the polymerization medium; although, higher polydispersities are observed in heterogeneous copper-mediated ATRP due to the lower concentration of the Cu (II) complex (deactivator) and consequently a slower deactivation process \[^{[12]}\].

Generally, more electron donating ligands better stabilize the higher oxidation state of the metal and accelerate the polymerization: nitrogen-based ligands generally work well for Cu-mediated ATRP; in contrast, sulfur, oxygen, or phosphorus ligands are less effective due to the different electronic effects or unfavourable binding constants \[^{[28],[38]}\].

The general order of activities of Cu complexes is related to their structure and follows the following order: tetradeutate (cyclic-bridged) \(\rightarrow\) tetradeutate (branched) \(\rightarrow\) tetradeutate (cyclic) \(\rightarrow\) trideutate \(\rightarrow\) tetradeutate (linear) \(\rightarrow\) bidentate ligands \[^{[39]}\]; Cu complexes formed with monodentate nitrogen based ligands do not promote successful ATRP.

However bidentate ligands, such as \(N\)-alkyl-(2-pyridyl) methanimine ligands \([N-(n-propyl) pyridylmethanimine (NPPMI) and N-(octyl) pyridylmethanimine (NOPMI)]\[^{[31]}\]\ and 2,2-bipyridine-based ligands \([2,2'\text{-bipyridyne (byp), 4,4'\text{-di(9-heptadecyl)-2,2'}\text{-bipyridyne (dHDbpy) \[^{[40]}\], 4,4'\text{-di(5-nonyl)-2,2'}\text{-bipyridyne (dNbpy) \[^{[41]}\] are commonly used in ATRP \[^{[9],[42],[43]}\], leading to well-controlled polymerizations but to relatively slow polymerization rates; moreover, the incorporation of long alkyl groups onto pyridine rings makes the bpy ligands (dHDbpy and dNbpy) more soluble in less polar solvents increasing complexes activity.

**Figure 1-4.** Chemical structures of various nitrogen-based ligands for copper complexes: bidentate ligands [a. 2,2-bipyridine-based ligands, b. \(N\)-alkyl-(2-pyridyl) methanimine ligands and c. \(N,N,N,N\)-tetramethylethylenediamine \((n=1), (\text{TMEDA})\)], tridentate ligands [c. \(N,N,N,N\)-pentamethyldiethylenetriamine \((n=2), (\text{PMDETA})\)], linear tetradentate [c. 1,1,4,7,10,10-Hexamethyltriethylenetetramine \((n=3), (\text{HMTETA})\)], branched tetradentate [d. Tris(2(dimethylamino)ethyl)amine(\(Me_2\text{TREN})\] and cyclic tetradentate [e. 1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane (Me\(_2\text{Cyclam})\] ligands.
1.3.2.5. Deactivator

The deactivator in ATRP is the higher oxidation state metal complex formed after atom transfer, and it plays a crucial role in ATRP in reducing the polymerization rate and the polydispersity of the final polymer; during the reaction the concentration of deactivator, continuously but slowly, increases in concentration with monomer conversion due to the persistent radical effect: propagating radicals $R\cdot M_n\cdot$ are rapidly trapped in the deactivation process by the deactivator species $[\text{Cu(II)LnX}]$, forming the dormant species which are consequently activated again by an appropriate catalyst to reform the growing centres from which polymer chains would continue to propagate.

Radicals can propagate ($k_p$) but also irreversibly terminate ($k_t$); however, persistent radicals ($X$) cannot terminate with each other but only (reversibly) cross-couple with the growing species ($k_d$); thus, every act of radical-radical termination is accompanied by the irreversible accumulation of $[\text{Cu(II)LnX}]$. The reversible deactivation step of the ATRP equilibrium is thus of primary importance and its rate is given by:

$$R_d = k_d [R\cdot][\text{Cu(II)LnX}] \quad \text{Eq. 1-5}$$

While the final molecular weights do not depend upon the concentration of deactivator, the polydispersity (eq. 1-4) and the rate of polymerization (eq. 1-2) will decrease with its increasing concentration.

1.3.2.6. Solvent

The main role of the solvent is essentially to solubilise the monomer, the initiator, the catalyst system and the polymer, which may be insoluble in its monomer.

Solvent choice should be dictated by several factors: chain transfer to solvent, which depends on the transfer constant ($C_s$), should be minimal; solvent interactions with the catalyst system should also be considered: first of all the structure of the catalyst (therefore its activity) may change depending on the solvent nature; furthermore
solvent-catalyst interactions may lead to solvolysis of the halogen ligand or catalyst poisoning (e.g., carboxylic acids or phosphine in copper based ATRP) \[32\]; solvent-assisted side reactions, such as elimination of HX from polystyryl halides at 110°C-130°C in a polar solvent \[44\], should be also minimized. Moreover reaction medium should be oxygen-free as the system is quite oxygen-sensitive: ATRP will proceed with small oxygen traces, because the oxygen can be scavenged by the catalyst, which is present at much higher concentration than the polymeric radicals; however oxidation of the catalyst by oxygen reduces the metal complex concentration potentially forming a substantial excess of deactivator and thus reducing the rate of polymerization.

1.3.2.7. Temperature and reaction time

The optimal temperature depends mostly on the monomer, the catalyst and the targeted molecular weight; in general, the solubility of the catalyst increases at higher temperatures; however, catalyst decomposition may also occur with the temperature increase \[45\], \[46\].

It is widely experienced that rate of polymerization in ATRP increases with increasing temperature due to the increase of both the radical propagation rate constant and the atom transfer equilibrium constant; as a result of the higher activation energy for the radical propagation than for the radical termination, higher $k_p/k_t$ ratios and better control (“livingness”) may be observed at higher temperatures. However, as mentioned in the previous paragraph, chain transfer and other side reactions become more pronounced at elevated temperatures.

The most important effect of reaction time in ATRP occurs at high conversions: at high monomer conversions, the rate of propagation would slow down considerably. Although the rate of any side reaction does not change significantly, as most of them are monomer concentration independent, prolonged reaction times leading to nearly complete monomer conversion may not increase the polydispersity of the final polymer but will induce loss of end groups \[47\]. Thus, to obtain polymers with high end-group
functionality or to subsequently synthesize block copolymers, conversion must not exceed 95% to avoid end-group loss.

In light of these considerations a unique combination of initiator, metal, ligands, deactivator, temperature, reaction time, and solvent must be employed for the ATRP of each particular monomer in order to obtain well-defined polymers.

1.4 Colloids

1.4.1 Colloid stability

A “colloidal system” can be defined as substance microscopically dispersed evenly throughout another one [48]; it consists of two separate phases: a dispersed phase (or internal phase) and a continuous phase (or dispersion medium) and it may be solid, liquid or gaseous.

Colloid systems usually have dimensions of less than one micron, which results in a very high surface-to-volume ratio. Homogeneous mixtures with a dispersed phase in this size range may be called colloidal aerosols, colloidal emulsions, colloidal foams, colloidal dispersions, or hydrosols. The dispersed-phase particles or droplets are affected largely by the surface chemistry present in the colloid.

Since the size of the dispersed phase may be difficult to measure and colloids have the appearance of solutions, colloids can be identified and characterized by their physico-chemical properties; in the interaction of colloid particles the following forces play an important role: A) excluded volume repulsion, thus the impossibility of any overlapping between hard particles; B) electrostatic interaction, as colloidal particles often carry an electrical charge therefore attract or repel each other; the charge of both the continuous and the dispersed phase, as well as the mobility of the phases are factors affecting this interaction; C) van der Waals forces due to interaction between two dipoles that are either permanent or induced. Even if the particles do not have a permanent dipole, fluctuations of the electron density gives rise to a temporary dipole in a particle. This temporary dipole induces a dipole in particles nearby. The temporary dipole and the
induced dipoles are then attracted to each other; D) entropic forces, according to the second law of thermodynamics, a system progresses to a state in which entropy is maximized. This can result in effective forces even between hard spheres; E) steric forces between polymer-covered surfaces or in solutions containing non-adsorbing polymer that can modulate interparticle forces, producing an additional steric repulsive force (which is predominantly entropic in origin) or an attractive depletion force between them.

Colloidal stability depends thus on the nature of the aforementioned forces: a system is colloidally stable when the particles do not aggregate at a significant rate: the precise connotation depends on the type of aggregation under consideration. An aggregate is, in general, a group of particles (which may be atoms or molecules) held together in any way: a colloidal particle itself (e.g. a micelle) may be regarded as an aggregate or more specifically aggregate is used to describe the structure formed by the cohesion of colloidal particles.

When a solution is collooidally unstable (i.e. the rate of aggregation is not negligible) the formation of aggregates is called coagulation or flocculation: coagulation implies the formation of compact aggregates leading to the macroscopic separation of a coagulum; flocculation implies the formation of a loose or open network which may or may not separate macroscopically. The reversal of coagulation or flocculation, i.e. the dispersion of aggregates to form a colloidaly stable suspension or emulsion, is called deflocculation (sometimes peptization).

The rate of aggregation is in general determined by the frequency of collisions and the probability of cohesion during collision. If the collisions are caused by Brownian motion, the process is called perikinetic aggregation; if by hydrodynamic motions (e.g. convection or sedimentation) one may speak of orthokinetic aggregation.

In hydrophobic solutions, coagulation can be brought about by changing the electrolyte concentration to the critical coagulation concentration (c.c.c.) which depends to some extent on the experimental circumstances (method of mixing, time between mixing and determining the state of coagulation, criterion for measuring the degree of coagulation, etc.). Moreover, addition of small amounts of a hydrophilic colloid to hydrophobic
solutions may make the latter more sensitive to flocculation by electrolyte. This phenomenon is called *sensitization*. Higher concentrations of the same hydrophilic colloid usually protect the hydrophobic solution from flocculation. This phenomenon is called *protective action*.

If the concentration of particles is high and interparticle forces are strong enough, suspended particles may tend to sediment under the action of gravity or a centrifugal field; this process of *sedimentation* may be better described as compaction of the particle structure with pressing out of the liquid (also called *subsidence*). *Sediment* is thus the highly concentrated suspension which may be formed by the sedimentation of a dilute suspension.

Coalescence may also occur in case of disappearance of the boundary between two particles (usually droplets or bubbles) in contact, or between one of these and a bulk phase followed by changes of shape leading to a reduction of the total surface area; if coalescence is extensive it leads to the formation of a macrophase and the emulsion is said to *break*.

Stabilization serves thus to prevent colloids from aggregating: steric and electrostatic stabilization are the two main mechanisms for colloid stabilization. Steric stabilization involves the use of polymers added to the system and adsorbed onto the particle surface that prevent the particle surfaces coming into close contact. If enough polymer adsorbs, the thickness of the coating is sufficient to keep particles separated by steric repulsions between the polymer layers and at those separations the van der Waals forces are too weak to cause the particles to adhere. Steric stabilization is simple, requiring just the addition of a suitable polymer. However it can be difficult to subsequently flocculate the system if this is required, the polymer can be expensive and in some cases the used polymer is undesirable e.g. when a ceramic slip is cast and sintered, the polymer has to be ‘burnt out’. This causes shrinkage and can lead to defects.

Electrostatic or charge stabilization is based on the mutual repulsion of like electrical charges. In general, different phases have different charge affinities, so that an *electrical double layer* (see section 1.4.2) forms at any interface. Small particle sizes lead to enormous surface areas, and this effect is greatly amplified in colloids. In a stable
colloid, mass of a dispersed phase is so low that its kinetic energy is too weak to overcome the electrostatic repulsion between charged layers of the dispersing phase. The charge on the dispersed particles can be observed by applying an electric field: all particles migrate to the same electrode and therefore must all have the same sign charge.

Unstable colloidal dispersions form flocs as the particles aggregate due to interparticle attractions. In this way photonic glasses can be grown. This can be accomplished by a number of different methods: 1) by the addition of salt to a suspension or changing the pH of a suspension to effectively neutralize or "screen" the surface charge of the particles in suspension; this removes the repulsive forces that keep colloidal particles separate and allows for coagulation due to van der Waals forces. 2) by the addition of polymer flocculants that can bridge individual colloidal particles by attractive electrostatic interactions or by addition of non-adsorbed polymers called depletants that cause aggregation due to entropic effects.

Unstable colloidal suspensions of low-volume fraction form clustered liquid suspensions, wherein individual clusters of particles fall to the bottom of the suspension (or float to the top if the particles are less dense than the suspending medium) once the clusters are of sufficient size for the Brownian forces that work to keep the particles in suspension to be overcome by gravitational forces. However, colloidal suspensions of higher-volume fraction form colloidal gels with viscoelastic properties, i.e. bentonite or toothpaste, that flow like liquids under shear, but maintain their shape when shear is removed.

1.4.2 DLVO theory

The scientists Derjaguin, Verwey, Landau and Overbeek developed a theory in the 1940s which dealt with the stability of colloidal systems: DLVO theory suggests that the stability of a particle in solution is dependent upon its total potential energy $V_T$. This theory recognizes that $V_T$ is the balance of several competing contributions:

$$V_T = V_A + V_R + V_S \quad \text{eq. 1-6}$$
where $V_S$ is the potential energy due to the solvent, it usually only makes a marginal contribution to the total potential energy over the last few nanometers of separation. Much more important is the balance between $V_A$ and $V_R$, the attractive and repulsive contributions: they potentially are much larger and operate over a much larger distance:

$$V_A = - \frac{A}{(12 \pi D^2)} \quad \text{eq. 1-7}$$

where $A$ is the Hamaker constant and $D$ is the particle separation. The repulsive potential $V_R$ is a far more complex function:

$$V_R = 2 \pi \varepsilon \varepsilon_0 r \xi^2 \exp(- \kappa D) \quad \text{eq. 1-8}$$

where $r$ is the particle radius, $\pi$ the solvent permeability, $\varepsilon$ the dielectric constant of the solvent, $\varepsilon_0$ the vacuum permittivity, $\kappa$ a function of the ionic composition ($\kappa^{-1}$ is the Debye screening length, a characteristic length of the Electric Double Layer) and $\xi$ is the zeta potential.

DLVO theory suggests that the stability of a colloidal system is determined by the sum of these van der Waals attractive ($V_A$) and electrical double layer repulsive ($V_R$) forces that exist between particles as they approach each other due to the Brownian motion they are undergoing. This theory proposes that an energy barrier resulting from the repulsive force prevents two particles approaching one another and adhering together. But if the particles collide with sufficient energy to overcome that barrier, the attractive force will pull them into contact where they adhere strongly and irreversibly together. Therefore if the particles have a sufficiently high repulsion, the dispersion will resist flocculation and the colloidal system will be stable; on the contrary if a repulsion mechanism does not exist then flocculation or coagulation will eventually take place.
Figure 1-5. Schematic diagrams of the variation of free energy with particle separation according to DLVO theory. Left: the net energy is given by the sum of the double layer repulsion and the van der Waals attractive forces that the particles experience as they approach one another. Right: at higher salt concentrations free energy may have a primary and a possible secondary minimum (diagrams from www.malvern.com).

Under conditions (e.g. high salt concentrations) that reduce the absolute value of ζ-potential, there is a possibility of developing a “secondary minimum” where a weak and potentially reversible adhesion between particles occurs (figure 1-5, right). The resulting flocs are sufficiently stable not to be broken up by Brownian motion, but may dissociate under an externally applied force such as vigorous agitation. Therefore to maintain the stability of the colloidal system, the repulsive forces must be dominant.

1.4.3 Diffuse layer and zeta potential

As most colloidal dispersions in aqueous media carry an electric charge, dissociation of acidic groups on the surface of a particle will give rise to a negatively charged surface; conversely, a basic surface will take on a positive charge. In both cases, the magnitude of the surface charge depends on the acidic or basic strengths of the surface groups and on the pH of the solution. The surface charge can be reduced to zero by suppressing the surface ionisation by decreasing the pH in case of negatively charged particles or by increasing the pH in the case of positively charged particles.
Therefore the development of a net charge at the particle surface affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions, ions of opposite charge to that of the particle, close to the surface. Thus an electrical double layer (EDL) or double layer (DL) exists round each particle.

The DL is composed of two parallel layers of charge surrounding the object. The first layer, the surface charge (either positive or negative), comprises ions adsorbed directly onto the object due to a host of chemical interactions. The second layer is composed of ions attracted to the surface charge via the Coulomb force, which electrically screen the first layer. This second layer is loosely associated with the object, because it is made of free ions which move in the fluid under the influence of electric attraction and thermal motion rather than being firmly anchored. It is thus called the diffuse layer.

Interfacial DL is usually most apparent in systems with a large ratio of surface area to volume, such as colloid or porous bodies with particles or pores (respectively) on the scale of micrometres to nanometres. However, the importance of DLs extends to other systems, e.g., DL is fundamental to the electrochemical behaviour of electrodes.

The earliest model of the electrical DL is usually attributed to Helmholtz \([49]\); the author treated the DL mathematically as a simple capacitor, based on a physical model in which a single layer of ions is adsorbed at the surface.

Later Louis Georges Gouy \([50, 51]\) and David Leonard Chapman \([52]\) made significant improvements by introducing a diffuse model of the electrical DL, in which the electric potential decreases exponentially away from the surface to the fluid bulk.

The Gouy-Chapman model fails for highly charged DLs. In order to resolve this problem Stern suggested the combination of the Helmholtz and Gouy-Chapman models, giving an internal Stern layer \([53]\) (also known as Helmholtz layer), and an outer diffuse layer (i.e. Gouy-Chapman layer). The combined Gouy-Chapman-Stern model is the most commonly used one. It still has some limitations, such as: 1) ions are effectively modelled as point charges; 2) the only significant interactions in the diffuse layer are coulombic; 3) dielectric permittivity is assumed constant throughout the double layer; 4) the viscosity of the fluid is constant above the slipping plane.
As stated by Lyklema, "...the reason for the formation of a “relaxed” (“equilibrium”) double layer is the non-electric affinity of charge-determining ions for a surface..." \[54\]. This process leads to the build up of an electric surface charge, expressed usually in C/m², which creates an electrostatic field which then affects the ions in the bulk of the liquid. This electrostatic field, in combination with the thermal motion of the ions, creates a counter charge, and thus screens the electric surface charge. The net electric charge in this screening diffuse layer is equal in magnitude to the net surface charge, but has the opposite polarity. As a result the complete structure is electrically neutral.

The diffuse layer, or at least part of it, can move under the influence of tangential stress. There is a conventionally introduced slipping plane that separates mobile fluid from
fluid that remains attached to the surface. The electric potential at this plane is called electrokinetic potential or zeta potential, also denoted as $\zeta$-potential.

The electric potential on the external boundary of the Stern layer versus the bulk electrolyte is referred to as Stern potential. Electric potential difference between the fluid bulk and the surface is called the electric surface potential.

The magnitude of the $\zeta$-potential gives an indication of the potential stability of the colloidal system: if all the particles in suspension have a large negative or positive $\zeta$-potential then they will tend to repel each other and there is no tendency to flocculate. However, if the particles have low $\zeta$-potential values then there is no force to prevent the particles coming together and flocculating. The general dividing line between stable and unstable suspensions is generally taken at either +30mV or -30mV. Particles with $\zeta$-potentials more positive than +30mV or more negative than -30mV are normally considered stable.

In aqueous media, the pH of the sample is one of the most important factors that affect its zeta potential. A zeta potential value on its own without defining the solution conditions is a virtually meaningless number: in case of a particle in suspension with a negative $\zeta$-potential, if more alkali is added to this suspension then the particles tend to acquire more negative charge. If acid is added to this suspension then a point will be reached where the charge will be neutralised. Further addition of acid will cause a build up of positive charge. Therefore a $\zeta$-potential versus pH curve will be positive at low pH and lower or negative at high pH (see Figure 1-7).

There also may be a point where the plot passes through zero zeta potential: this point is called the isoelectric point and it is normally the point where the colloidal system is least stable.
The thickness of the double layer, called Debye length ($\kappa^{-1}$), depends upon the concentration of ions in solution and can be calculated from the ionic strength of the medium.

The higher the ionic strength, the more compressed the double layer becomes. The valency of the ions will also influence double layer thickness. A trivalent ion such as $\text{Al}^{3+}$ will compress the double layer to a greater extent in comparison with a monovalent ion such as $\text{Na}^+$. Inorganic ions can interact with charged surfaces in one of two distinct ways: (i) non-specific ion adsorption where they have no effect on the isoelectric point; (ii) specific ion adsorption, which will lead to a change in the value of the isoelectric point. The specific adsorption of ions onto a particle surface, even at low concentrations, can have a dramatic effect on the zeta potential of the particle dispersion. In some cases, specific ion adsorption can lead to charge reversal of the surface.

An important consequence of the existence of electrical charges on the surface of particles is that they interact with an applied electric field. These effects are collectively defined as electrokinetic effects. There are four distinct effects depending on the way in which the motion is induced:
1) *Electrophoresis*: the movement of a charged particle relative to the liquid suspended in under the influence of an applied electric field.

2) *Electroosmosis*: the movement of a liquid relative to a stationary charged surface under the influence of an electric field.

3) *Streaming potential*: the electric field generated when a liquid is forced to flow past a stationary charged surface.

4) *Sedimentation potential*: the electric field generated when charged particles sediment.

When an electric field is applied across an electrolyte, charged particles suspended in the electrolyte are attracted towards the electrode of opposite charge. Viscous forces acting on the particles tend to oppose this movement. When equilibrium is reached between these two opposing forces, the particles move with constant velocity. The velocity is dependent on the strength of electric field or voltage gradient, the dielectric constant of the medium, the viscosity of the medium and the ζ-potential. The velocity of a particle in a unit electric field is referred to as its electrophoretic mobility. Thus ζ-potential is related to the electrophoretic mobility by the Henry equation:

\[
U_E = \frac{2 \varepsilon \zeta f(\kappa_o)}{3 \eta} \quad \text{eq. 1-9}
\]

where \(U_E\) is electrophoretic mobility, \(\zeta\) the zeta potential, \(\varepsilon\) the dielectric constant, \(\eta\) the viscosity and \(f(\kappa_o)\) the Henry’s function.

The parameter \(\kappa_o\) refers to the radius of the particle and therefore measures the ratio of the particle radius to electrical double layer thickness. Electrophoretic determinations of ζ-potential are most commonly made in aqueous media and moderate electrolyte concentration. In this case is 1.5, and this is referred to as the Smoluchowski approximation. Therefore calculation of zeta potential from the mobility is straightforward for systems that fit the Smoluchowski model, i.e. particles larger than about 0.2 microns dispersed in electrolytes containing more that \(10^{-3}\) molar salt.

For small particles in low dielectric constant media (eg non-aqueous media), becomes 1.0 and allows an equally simple calculation. This is referred to as the Huckel approximation. The essence of a classical microelectrophoresis system is a capillary cell with electrodes at either end to which a potential is applied. Particles move towards the
electrode, their velocity is measured and expressed in unit field strength as their mobility.

1.4.4 Dynamic light scattering

Dynamic Light Scattering (DLS), also called Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering, is a technique for measuring the size of particles typically in the sub micron region.

DLS measures Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them.

Normally DLS is concerned with measurement of particles suspended within a liquid. The larger the particle, the slower the Brownian motion will be. Smaller particles are “kicked” further by the solvent molecules and move more rapidly. An accurately known temperature is necessary for DLS because knowledge of the viscosity is required (because the viscosity of a liquid is related to its temperature).

The temperature also needs to be stable, otherwise convection currents in the sample will cause non-random movements that will ruin the correct interpretation of size.

The velocity of the Brownian motion is defined by a property known as the translational diffusion coefficient (D) and the size of a particle is thus calculated from the translational diffusion coefficient by using the Stokes-Einstein equation:

\[ d(H) = \frac{(k \ T)}{3 \ \pi \ \eta \ D} \]  

where \(d(H)\) is hydrodynamic diameter, \(D\) the translational diffusion coefficient, \(k\) Boltzmann’s constant, \(T\) the absolute temperature and \(\eta\) the viscosity.

As the diameter measured in DLS is a value that refers to how a particle diffuses within a fluid it is thus referred to as hydrodynamic diameter. The translational diffusion coefficient will depend not only on the size of the particle “core”, but also on any surface structure, as well as the concentration and type of ions in the medium.
Ionic concentration can affect the particle diffusion speed by changing the thickness of the electric double layer called the Debye length ($\kappa^{-1}$). Thus a low conductivity medium will produce an extended double layer of ions around the particle, reducing the diffusion speed and resulting in a larger, apparent hydrodynamic diameter. Conversely, conductivity media will suppress the electrical double layer and the measured hydrodynamic diameter.

Moreover any change to the surface of a particle that affects the diffusion speed will correspondingly change the apparent size of the particle. An adsorbed polymer layer projecting out into the medium will reduce the diffusion speed more than if the polymer is lying flat on the surface. The nature of the surface and the polymer, as well as the ionic concentration of the medium can affect the polymer conformation, which in turn can change the apparent size by several nanometres.

In dynamic light scattering, the diffusion speed of the particles is detected by measuring the rate at which the intensity of the scattered light fluctuates using a suitable optical arrangement: when a cuvette containing stationary particles is illuminated by a laser and a frosted glass screen is used to view the sample cell, a classical speckle pattern would be seen (see Figure 1-8). The speckle pattern will be stationary both in speckle size and position because the whole system is stationary.

![Schematic representation of a speckle pattern.](image)

**Figure 1-8.** Schematic representation of a speckle pattern.

For a system of particles undergoing Brownian motion, a speckle pattern is thus observed where the position of each speckle is seen to be in constant motion. This is because the phase addition from the moving particles is constantly evolving and
forming new patterns. The rate at which these intensity fluctuations occur will depend on the size of the particles: small particles cause the intensity to fluctuate more rapidly than the large ones. It is possible to directly measure the spectrum of frequencies contained in the intensity fluctuations arising from the Brownian motion of particles, but the best way is to use a signal comparator, generally referred to as digital autocorrelator.

It is designed to measure the degree of similarity between two signals, or one signal with itself at varying time intervals; if the intensity of a signal is compared with itself at a particular point in time and a time much later, there will be no correlation between the two signals, but if the intensity of signal at time $t$ is compared to the intensity a very small time later $(t+\delta t)$ (usually nanoseconds or microseconds), there will be a strong relationship or correlation between the intensities of two signals.

However if the signal, derived from a random process such as Brownian motion, at time $t$ is compared to the signal at $t+2\delta t$, there will still be a reasonable comparison or correlation between two signals, but it will not be as good as the comparison at $t$ and $t+\delta t$ because the correlation is reducing with time.

If the signals at $t+2\delta t$, $t+3\delta t$, $t+4\delta t$ etc. are compared with the signal at $t$, the correlation of a signal arriving from a random source will decrease with time until at some time, effectively $t = \infty$, there will be no correlation. In case particles are large the signal will be changing slowly and the correlation will persist for a long time while if the particles are small and moving rapidly then correlation will reduce more quickly.

The time at which the correlation starts to significantly decay is thus an indication of the mean size of the sample: the steeper the line, the more monodisperse the sample is and conversely, the more extended the decay becomes, the greater the sample polydispersity.

The correlator used in the instrument will thus construct the correlation function $G(\tau)$ of the scattered intensity:

$$G(\tau) = < I(t).I(t+\tau) > \quad \text{eq. 1-11}$$

Where $\tau$ is the time difference (the sample time) of the correlator.

For a large number of monodisperse particles in Brownian motion, the correlation function is an exponential decaying function of the correlator time delay $\tau$: 
where $A$ is the baseline of the correlation function, $B$ intercept of the correlation function and $\Gamma = q^2 D$, where $D$ is the translational diffusion coefficient; moreover $q = \left(\frac{4 \pi n}{\lambda_0}\right) \sin(\theta/2)$, where $n$ is refractive index of dispersant, $\lambda_0$ the wavelength of the laser, $\theta$ the scattering angle.

For polydisperse samples, the equation can be written as:

$$G(\tau) = A[1 + B g_1(\tau)]^2 \quad \text{eq. 1-13}$$

where $g_1(\tau) = \text{is the sum of all the exponential decays contained in the correlation function.}$

Size is thus obtained from the correlation function by using various algorithms.

There are two approaches that can be taken: 1) fitting a single exponential to the correlation function to obtain the mean size (z-average diameter) and an estimate of the width of the distribution (polydispersity index), called the Cumulants analysis.

2) Fitting a multiple exponential to the correlation function to obtain the distribution of particle sizes, such as Non-negative least squares (NNLS) or CONTIN.

The size distribution obtained is a plot of the relative intensity of light scattered by particles in various size classes and it is therefore known as an intensity size distribution.

### 1.5 Nanostructured materials

#### 1.5.1 Polymer brushes at interfaces

Progress in polymer synthesis achieved in recent years brings polymer materials science closer to the goal of crafting materials with purposefully designed functional
nanostructures, *e.g.* by utilizing self-assembly in well defined block copolymers or through synthesis of complex nanoscale objects, acting as building blocks of even more complex, functional materials [55], [56], [57], [58], [59]. Such engineering of nanostructures based on macromolecular engineering appears as a particularly attractive strategy for future nanotechnologies.

In the past decade the use of controlled/living radical polymerization to prepare nanostructured materials has gained significant attention, as a wider range of functional monomers can be incorporated into polymeric and composite systems [6], [60], [61]. Among these, polymer brushes re-emerged recently as a particularly fascinating synthetic target [62], [63], [64].

Polymer brushes are defined as dense layers of chains confined to a surface or interface where the distance between grafts is much less than the unperturbed dimensions of the tethered polymer. Due to the high steric crowding, grafted chains extend from the surface and reside in an entropically unfavorable conformation. The brushes can be achieved by the end-grafting of chains to/from flat or curved surfaces that are organic or inorganic in nature: these include functional colloids, highly branched polymers and block copolymer aggregates, such as micelles or phase-separated nanostructures.

Generally, there are two ways to fabricate polymer brushes grafting polymer chains onto a solid surface: physisorption and covalent attachment.

Physisorption is a reversible process and is achieved by the self-assembly of polymeric surfactants or end-functionalized polymers on the surface. The surface grafting density and all other characteristic dimensions of the structure are controlled by thermodynamic equilibrium, albeit with possible different kinetics [55].

Physisorption of block copolymers or graft copolymers at an interface occurs through the selective solvation and selective adsorption, respectively [64]. The detailed polymer brush structure depends on the selectivities of these media and the nature of the copolymers, the architecture of copolymers, the length of each block and the interactions between blocks and surface. In the case of selective solvents [65], an ideal solvent is a precipitant for one block which forms an “anchor” layer on the surface and a good solvent for other block which forms polymer brushes in the solution. In the case of
a selective surface \[66\], one block is preferentially adsorbed on the surface and another one forms a polymer brush.

Nonetheless, the physisorption approach implies various preparative and purification steps as it requires the \textit{ex situ} preparation and the subsequent isolation of the macromolecular material; moreover weak interactions between the substrate and the block copolymer can lead to thermal and solvolytic instabilities. This drawback can be overcome by covalently attaching polymer chains to the substrate.

Covalent attachment can be accomplished by either “grafting to” or “grafting from” approaches: the “grafting to” approach is based on the use of preformed, end-functionalized polymers that react with a suitable substrate surface under appropriate conditions to form a tethered polymer brush. The covalent bond formed between surface and polymer chain makes the polymer brushes robust and resistant to common chemical environmental conditions. This method has been used often in the preparation of polymer brushes. End-functionalized polymers with a narrow molecular weight distribution can be synthesized by living anionic, cationic, radical, group transfer and ring opening metathesis polymerizations \[64\]. The substrate surface also can be modified to introduce suitable functional groups by coupling agents or self-assembled monolayers (SAMs). However, as the grafting density increases, it becomes increasingly harder for new chains to diffuse to the surface and react with it, leading to low surface graft densities. This limitation can be overcome by utilizing the “grafting from” approach where surface immobilized initiators start the polymerization and monomer adds to the growing polymer brushes with small influence of dimensions and mutual sterical constraints of the grafting material onto the polymer surface density that depends on the initial surface density of the initiating groups.

Although “grafted to” polymers are generally better defined and characterized, because they can be first isolated and purified then grafted, “grafting from” technique is a more promising method in the synthesis of polymer brushes because it provides higher grafting density and structures with low molecular weight dispersion and end functionalization, hence the possibility of block copolymerization \[57\].

All controlled free radical polymerization methods, namely ATRP \[67\], \[68\], \[69\], \[70\] RAFT \[61\], nitrooxide-mediated free radical polymerization (NMP) \[71\], \[72\], \[73\] and the
polymerization method based on iniferters have been applied to grow polymer films on the surface of a substrate by grafting the initiator directly to the surface. While the method of iniferters is plagued by high polydispersities and poor control over the functional groups at the chain ends the TEMPO-mediated polymerization has been used with great success, but its application is limited only to styrenic monomers or their mixtures with other monomers. RAFT is a promising technique because of the great number of monomers that can be successfully polymerized in a controlled manner, but the occurrence of transfer reactions to solution with the formation of soluble polymer limits the applicability of surface-initiated RAFT polymerizations. ATRP has been demonstrated to be a versatile technique to synthesize well-defined brushes (co)polymers, as well as complex (co)polymers and organic/inorganic hybrid materials, due to the following features: the polymerization can also be performed in very mild conditions (room temperature in aqueous solutions), with high yield and on a broad range of monomers; furthermore the occurrence of transfer reactions (with polymer production in solution) is negligible, because the radical species are always present at the end of the growing surface-tethered polymer chains, thus controlling film thickness and properties by the surface initiated growth of well-defined (co)polymers.

The modulation of polymer-brush composition and degree of polymerization (DP) using ATRP has been extensively investigated to modify surface properties to create nanopatterns and to design stimuli-responsive materials. ATRP of various monomers from preformed polymeric or colloidal initiators has been achieved yielding nano-objects of precise dimension and functionality. ATRP initiator groups have been successfully coated onto both organic and inorganic materials, with either flat or curved surfaces. From this approach, polymer brushes of varying composition and dimensions have been prepared by surface-initiated growth from macroscopic wafers or particles, (sub) micron-sized colloids and polymer backbones as represented in scheme 1-2.
Scheme 1-2. Examples of polymer brushes synthesized by ATRP using ‘‘grafting from’’ approach from various functional substrates, such as flat wafers, particles, colloids and polymers (X-halogen) [95].

The synthesis of brushes on particle surfaces has been widely conducted to prepare solid supports, chromatographic stationary phases, or high surface area for brushes on flat surfaces. Brushes were grown by surface-initiated ATRP, from organic latex colloids emulsions, shells of shell-crosslinked micelles, and from functionalized inorganic particles, such as silica, gold, alumina, polysilsesquioxane, titanium oxide clusters, iron oxides and germanium [95].

Dense brush layers on flat wafers and surfaces are among the most extensively investigated systems, owing to potential applications in advanced microelectronics and biotechnology: the main challenge in ATRP from flat surfaces with very low concentrations of initiating groups stems from the fact that, after halogen atom transfer to the transition metal catalyst, the concentration of persistent radical (deactivator) may be too low to reversibly trap the propagating radicals, leading to uncontrolled chain growth. This challenge can be effectively addressed through the addition of a persistent
radical (deactivator), or “sacrificial initiator”, at the beginning of the reaction facilitating the exchange reactions between active radicals and dormant oligo/polymeric halides: in these reactions only surface-bound alkyl halides were employed as initiators and linear polymers were not formed in solution. Identical surface-initiated ATRP performed without the addition of deactivator resulted in the rapid polymerization and termination of tethered polymeric chains, where film thickness did not increase despite extended reaction times.

One of the difficulties in assessing the living character of surface-initiated ATRP from flat surfaces is the low mass of tethered polymers attached to the substrate.

The addition of untethered small molecule initiators to ATRP mixtures with functional flat substrates serves a number of beneficial purposes in both synthesis and characterization of polymer brushes.

In systems with added free initiator, sufficient concentrations of persistent radical (deactivator) are generated by the termination of radicals formed in solution. Furthermore, the final DP of tethered chains on surfaces can be dictated by the concentration of sacrificial initiator added at the initial stages of the polymerization. The determination of both monomer conversion and molar mass of polymers in the system is also greatly facilitated as the analysis of free polymers formed in solution can be performed by standard techniques, such as $^1$H-NMR spectroscopy, GC and GPC. Although molar masses of tethered polymers from flat brushes and free polymers prepared by ATRP in the presence of sacrificial initiator have not yet been compared, GPC analysis of cleaved polymers from brushes prepared from silica particles has shown that cleaved chains from surfaces have similar molar masses and polydispersities to polymers formed in solution.$^{[95]}$

The conformation of tethered chains and the film thickness in polymer-brush layers are directly affected thus by the surface coverage of the initiating groups on the flat surface.
1.5.2 Spherical brushes: silica nanoparticles

The use of ATRP initiator groups tethered onto inorganic nano-sized colloids and the subsequent growth by surface-initiated grafting of polymer chains of varying composition and dimensions produce hybrid nanoparticles: these materials, composed of an inorganic core and an organic polymer shell, have attracted growing interest due to the intriguing properties associated with the core (optical, magnetic, mechanical properties, etc.) and the desired properties of polymers (processability, compatibility to the environment, etc.). If the shell is composed of polymer chains that are densely tethered by one end via a covalent bond to the surface of a spherical core particle, hairy nanoparticles are obtained as polymer chains are forced to stretch away from the grafting sites achieving thus spherical brushes [96].

Among organic/inorganic hybrid nanoparticles, metal nanoparticles are increasingly gaining importance because of their unique size-dependent magnetic, optical, electrical, and catalytic properties: in effect polymer-assisted synthesis of metal nanoparticles has received considerable attention because of (1) the small concentrations of homopolymers and block copolymers capable of stabilizing nanoparticles effectively by steric stabilization, (2) the functional groups of the polymer serving as both reducing and stabilizing (capping) agent, (3) the easy variation of the core size of the nanoparticles by the variation of the polymer/metal salt ratio, and (4) the ease of preparation of metal–polymer nanocomposites.

However, one of the main issues in achieving well-defined and stable nanoparticles is the so called bridging flocculation due to interparticle aggregation of the nanoparticles: the two general types of stabilization to prevent aggregation are charge stabilization, thus an electrostatic stabilization based on repulsive electrostatic forces between charged surfaces, and steric stabilization, by grafting long polymer chains.

Schneider and Decher [97] reported in a study the experimental parameters for controlling particle aggregation and for enhancing dispersion stability of functional core/shell gold nanoparticles using electrostatic layer-by-layer assembly of certain polyelectrolytes, suggesting three possible regimes: regime I has colloids in excess of polyelectrolytes that results in incomplete surface coverage and only very few free...
polyion chains left in solution; regime II has colloids and polyelectrolytes in approximately equal charge proportion, known as the flocculation regime, and must completely be avoided; regime III has polyelectrolytes in excess of colloids that brings to excellent surface coverage and a very good stability of the suspension. In general, one should expect that in regime III adsorption is favoured over bridging flocculation if individual nanoparticles are sufficiently separated from each other (the nanoparticle concentration) and the length of the oppositely charged polyelectrolytes is sufficiently small (degree of polymerization). Similarly, organic/inorganic hybrid systems composed by silica nanoparticles for example, where the attraction between the particles (hydrophilic) is enhanced by the polymer matrix nature (generally hydrophobic), can be also stabilized sterically: the grafting of a dense layer of hydrophilic polymer chains, such as oligo (ethylene glycol) methacrylate (OEGMA), 2-hydroxyethyl methacrylate (HEMA) and glycerol monomethacrylate (GMMA), produced stable silica colloids and the aqueous solution properties of the grafted polymer chains determine the colloid stability of the particles [98].

Many authors have reported the surface-initiated polymerization by ATRP of Silica nanoparticles using a wide variety of monomers.

Nanoparticles possessing covalently bound pS (poly-styrene) chains were prepared by atom transfer radical polymerization of styrene from functionalized colloidal silica particles: in the synthesis of silica-graft-polystyrene (SiO$_2$-g-pS) spherical brushes, the ATRP of styrene was performed using bromoisobutyrate-functionalized silica colloids targeting varying DP of tethered chains; SiO$_2$-g-pS hybrid nanoparticles, characterized both in the solid state and in solution using transmission electron microscopy (TEM) and dynamic light scattering (DLS), possessed molar masses of tethered pS in the range of $M_n$ 14 000 to 33 000 g/mol [95].

In effect many authors have reported the ATRP of styrene, MMA [99, [100], [101], [102], [103], [104] and block copolymers of butyl acrylate-methyl methacrylate (BA-b-MMA) [95] (see Figure 1-5) from core-shell silica particles and demonstrated that it can be successfully performed with a high degree of control during the polymerization despite a deviation from theoretical molecular weights.
Moreover, Carrot et al. [105] reported the synthesis of atom transfer radical polymerization (ATRP) of different active initiators from well-defined silica nanoparticles and the use of the ATRP initiators in the grafting of poly(n-butyl acrylate) from the silica particle surface; the use of n-butyl acrylate as the monomer permits one to obtain nanocomposites with a hard core and a soft shell where film formation is facilitated. In other works it has been reported the use of this monomer copolymerized with pS in block copolymer brushes on the surfaces of intercalated and exfoliated silicate (clay) layers [106] or with poly(methyl methacrylate) for the preparation of a wide range of polymer brushes where (co)polymers are covalently attached to a flat surface of silica [95].

A number of homopolymers and functionalized block copolymers are useful for the preparation of functional nanoparticles. In general, water-soluble polymers such as poly[2-(N,N-dimethylamino)ethyl methacrylate] (PDMAEMA), poly(2-vinylpyridine)
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(PVP), poly(vinylalcohol) (PVA), and poly(acrylic acid) (pAA) are well known for their ability to coordinate onto metal particles. On the other hand, hydrophobic polymers such as PS, pMMA, and poly(t-butyl methacrylate) (PtBMA) are unable to coordinate with metal nanoparticles, and thus the introduction of a coordinating group is necessary for them to stabilize metal nanoparticles. To prepare polymer–metal nanocomposites with such hydrophobic polymers, suitable decoration of the metal nanoparticles is necessary: one approach consists of the in situ preparation of nanoparticles in the matrix either by the reduction of metal salts dissolved in the polymer matrix or by the evaporation of metals on the heated polymer surface; another approach is the polymerization of the matrix around the nanoparticles; a third useful approach is based on the blending of metal nanoparticles and polymers, both of whom are previously prepared: this technique provides full synthetic control over both the nanoparticles and the polymer matrix.

A range of polyelectrolyte-grafted silica particles have been prepared by grafting suitable initiators onto monodisperse silica particles using siloxane chemistry, followed by surface-initiated atom transfer radical polymerization (ATRP) of different ionic vinyl monomers, namely sodium 4-styrenesulfonate (SStNa), sodium 4-vinylbenzoate (NaVBA), 2-(dimethylamino)ethyl methacrylate (DMAEMA), and 2-(diethylamino)ethyl methacrylate (DEAMA) in protic media \[107\], \[108\], \[109\].

In principle, as previously mentioned, polyelectrolyte brushes attached to the surface of colloidal particles should enhance their stability against flocculation in aqueous media due to the additional steric/electrostatic repulsive forces: however the colloid stabilities of the polyelectrolyte-grafted silica particles are dictated by the nature of the grafted polyelectrolyte and are highly pH-dependent.

Therefore, through the use of stimuli-responsive polymers or polymeric systems, it is possible to prepare environmentally responsive (pH, temperature) hairy hybrid nanoparticles, which are attractive building blocks for design and fabrication of smart nanostructured materials.

A typical example of thermosensitive polymer brushes on silica nanoparticles is offered by the immobilization of hyper branched pS displaying a 2-bromoisobutyryl terminal
group as an initiator for atom transfer radical polymerization, which, once on the silica surface, produce grafted poly(N-isopropylacrylamide): pNIPAAm exhibits thermally reversible hydrophilic–hydrophobic changes across a lower critical solution temperature (LCST, 32 °C in aqueous solution) that can impart a responsive character to silica surfaces\textsuperscript{[110]}.

For many applications, the control of surface functionality is the key for controlling the nanoparticles interaction with biological species, self-assembly, dispersion in organic media, and compatibility with polymeric materials; for instance, magnetic nanoparticles functionalized with biocompatible macromolecules are potential for biomedical applications such as magnetic field assisted extraction of cells and macromolecules, site-specific drug delivery, gene analysis as well as magnetic hyperthermia in cancer treatment.

\section*{1.5.3 Sol-gel chemistry}

The sol-gel process, also known as chemical solution deposition, is a wet-chemical technique widely used in the field of materials science and ceramic engineering. Such methods are used primarily for the fabrication of materials (typically a metal oxide) starting from a chemical solution (or sol) which acts as the precursor for an integrated network (or gel) of either discrete particles or network polymers. Typical precursors are metal alkoxides and metal chlorides, which undergo various forms of hydrolysis and polycondensation reactions.

In this chemical procedure, the 'sol' (or solution) gradually evolves towards the formation of a gel-like diphasic system containing both a liquid phase and solid phase whose morphologies range from discrete particles to continuous polymer networks. The basic structure or morphology of the solid phase can range anywhere from discrete colloidal particles to continuous chain-like polymer networks\textsuperscript{[111, 112]}.

Under certain chemical conditions (typically in base-catalyzed sols), the particles may grow to sufficient size to become colloids, which are affected both by sedimentation and
forces of gravity. Stabilized suspensions of such sub-micrometer spherical particles may eventually result in their self-assembly, yielding highly ordered microstructures reminiscent of the prototype colloidal crystal which are called precious opal [113-115].

Under certain chemical conditions (typically in acid-catalyzed sols), the inter-particle forces have sufficient strength to cause considerable aggregation and/or flocculation prior to their growth. The formation of a more open continuous network of low density polymers exhibits certain advantages with regard to physical properties in the formation of high performance glass and glass/ceramic components in 2 and 3 dimensions [116].

In either case (discrete particles or continuous polymer network) the sol evolves then towards the formation of an inorganic network containing a liquid phase (gel). Formation of a metal oxide involves connecting the metal centers with oxo (M-O-M) or hydroxo (M-OH-M) bridges, therefore generating metal-oxo or metal-hydroxo polymers in solution. In both cases (discrete particles or continuous polymer network), the drying process serves to remove the liquid phase from the gel, yielding a micro-porous amorphous glass or micro-crystalline ceramic. Subsequent thermal treatment (firing) may be performed in order to favor further polycondensation and enhance mechanical properties.

In the case of the colloid, the volume fraction of particles (or particle density) may be so low that a significant amount of fluid may need to be removed initially for the gel-like properties to be recognized. This can be accomplished in any number of ways. The simplest method is to allow time for sedimentation to occur, and then pour off the remaining liquid. Centrifugation can also be used to accelerate the process of phase separation.

Removal of the remaining liquid (solvent) phase requires a drying process, which is typically accompanied by a significant amount of shrinkage and densification. The rate at which the solvent can be removed is ultimately determined by the distribution of porosity in the gel. The ultimate microstructure of the final component will clearly be strongly influenced by changes imposed upon the structural template during this phase of processing.
Afterwards, a thermal treatment, or firing process, is often necessary in order to favor further polycondensation and enhance mechanical properties and structural stability via final sintering, densification and grain growth. One of the distinct advantages of using this methodology as opposed to the more traditional processing techniques is that densification is often achieved at a much lower temperature.

The precursor sol can be either deposited on a substrate to form a film (e.g. by dip coating or spin coating), cast into a suitable container with the desired shape (e.g. to obtain monolithic ceramics, glasses, fibers, membranes, aerogels) or used to synthesize powders (e.g., microspheres, nanospheres).

The sol-gel approach is a cheap and low-temperature technique that allows for the fine control of the product’s chemical composition. Even small quantities of dopants, such as organic dyes and rare earth elements, can be introduced in the sol and end up uniformly dispersed in the final product. It can be used in ceramics processing and manufacturing as an investment casting material, or as a means of producing very thin films of metal oxides for various purposes. Sol-gel derived materials have diverse applications in optics, electronics, energy, space, biosensors, medicine (e.g. controlled drug release), reactive material and separation (e.g., chromatography) technology.

The interest in sol-gel processing can be traced back in the mid-1880s with the observation that the hydrolysis of tetraethyl orthosilicate (TEOS) under acidic conditions led to the formation of SiO₂ in the form of fibers and monoliths. Sol-gel research grew to be so important that in the 1990s more than 35,000 papers were published worldwide on the process [117-119].

The first step in a sol-gel reaction is the formation of an inorganic polymer by hydrolysis and condensation reactions, i.e., the transformation of the molecular precursor into a highly crosslinked solid. Hydrolysis leads to a sol, a dispersion of colloidal particles in a liquid, and further condensation results in a gel, an interconnected, rigid and porous inorganic network enclosing a continuous liquid phase: this transformation is called the sol-gel transition.

The sol-gel processes can be classified into two different routes depending on the nature of the precursors: a) the precursor is an aqueous solution of an inorganic salt or b) a
metal organic compound [120]. The inorganic route involves the formation of condensed species from aqueous solutions of inorganic salts by adjusting the pH, by increasing the temperature or by changing the oxidation state. But this method has several disadvantages. The aqueous chemistry of transition metal ions can be rather complicated because of the formation of a large number of oligomeric species, depending on the oxidation state, the pH or the concentration. The role of the counter anions, which are able to coordinate the metal ion giving rise to a new molecular precursor with different chemical reactivity towards hydrolysis and condensation, is almost impossible to predict. These ions can influence the morphology, the structure and even the chemical composition of the resulting solid phase. Also the removal of these anions from the final metal oxide product is often a problem. Many of these issues can be avoided by using metal alkoxides as precursors.

Metal alkoxides are members of the family of organometallic compounds with one or more metal atoms in the molecule. Metal alkoxides (R-O-M) are like alcohols (R-OH) with a metal atom, M, replacing the hydrogen H in the hydroxyl group. The formation of a metal oxide involves connecting the metal centers with oxo (M-O-M) or hydroxo (M-OH-M) bridges, therefore generating metal-oxo or metal-hydroxo polymers in solution. They are often soluble in organic solvents, providing high homogeneity, and they can easily be converted to the corresponding oxide.

The sol-gel conversion of metal alkoxides involves two main reaction types: hydrolysis and condensation. During hydrolysis, the alkoxide groups (-OR) are replaced via the nucleophilic attack of the oxygen atom of a water molecule under release of alcohol and the formation of a metal hydroxide. Condensation reactions between two hydroxylated metal species leads to M-O-M bonds under release of water (oxolation), whereas the reaction between a hydroxide and an alkoxide leads to M-O-M bonds under release of an alcohol (alkoxolation).

Chemical aspects play an important role in studying and controlling the sol-gel process. The chemical reactivity of metal alkoxides towards hydrolysis and condensation depends mainly on the electronegativity of the metal atom, its ability to increase the coordination number, the steric hindrance of the alkoxy group, and on the molecular structure of the metal alkoxides (monomeric or oligomeric). The amount of added water
in the hydrolysis step and how the water is added, determines, whether the alkoxides are completely hydrolyzed or not and which oligomeric intermediate species are formed. Additional parameters are the polarity, the dipole moment, and the acidity of the solvent.

However the major problem of sol-gel methods based on the hydrolysis and condensation of molecular precursors is the control over the reaction rates. For most transition metal oxide precursors, these reactions are too fast, resulting in loss of morphological and also structural control over the final oxide material. Furthermore, the different reactivities of metal alkoxides make it difficult to control the composition and the homogeneity of complex multimetal oxides by the sol-gel process. One possibility to decrease and to adjust the reactivity of the precursors is the use of organic additives like carboxylic acids, β-diketones or functional alcohols, which act as chelating ligands and modify the reactivity of the precursors \[120, 121\]. An alternative strategy involves the slow release of water by chemical or physical processes, allowing control over the local water concentration and thus, over the hydrolysis of the metal oxide precursors \[122\]. In spite of all these efforts, the strong sensitivity of aqueous sol-gel processes towards any slight changes in the synthesis conditions and the simultaneous occurrence of hydrolysis and condensation reactions make it still impossible to fully control the sol-gel processing of metal oxides in aqueous medium.

A well studied alkoxide is silicon tetraethoxide, or tetraethyl orthosilicate (TEOS). The chemical formula for TEOS is given by: Si(OC\(_2\)H\(_5\))\(_4\), or Si(OR)\(_4\) where the alkyl group R is C\(_2\)H\(_5\). In the reaction of hydrolysis a hydroxyl ion becomes attached to the silicon atom as follows:

\[
\text{Si(OR)}_4 + \text{H}_2\text{O} \rightarrow \text{HO-Si(OR)}_3 + \text{R-OH}
\]

Depending on the amount of water and catalyst present, hydrolysis may proceed to completion, so that all of the OR groups are replaced by OH groups, as follows:

\[
\text{Si(OR)}_4 + 4 \text{H}_2\text{O} \rightarrow \text{Si(OH)}_4 + 4 \text{R-OH}
\]

Any intermediate species \([(\text{OR})_2-\text{Si-(OH)}_2]\) or \([(\text{OR})_3-\text{Si-(OH)}]\) would be considered the result of partial hydrolysis. In addition, two partially hydrolyzed molecules can link together in a condensation reaction to form a siloxane [Si–O–Si] bond:
(OR)₃–Si–OH + HO–Si–(OR)₃ → [(OR)₃Si–O–Si(OR)₃] + H–O–H

or

(OR)₃–Si–OR + HO–Si–(OR)₃ → [(OR)₃Si–O–Si(OR)₃] + R–OH

Thus, polymerization is associated with the formation of a 1, 2, or 3-dimensional network of siloxane [Si–O–Si] bonds accompanied by the production of H–O–H and R–O–H species.

By definition, condensation liberates a small molecule, such as water or alcohol. This type of reaction can continue to build larger and larger silicon-containing molecules by the process of polymerization that can lead to complex branching of the polymer, because a fully hydrolyzed monomer Si(OH)₄ is tetrafunctional (can branch or bond in 4 different directions). Alternatively, under certain conditions (e.g., low water concentration) fewer than 4 of the OR or OH groups (ligands) will be capable of condensation, so relatively little branching will occur. This process of hydrolysis and condensation, and the factors that bias the structure toward linear or branched structures are the most critical issues of sol-gel science and technology[123-130].

The formation of silica particles can be divided into two stages: nucleation and growth. Generally, two different approaches can be used to describe particle formation and growth. The first is via addition of hydrolyzed monomers to the surface of the nuclei[131, 132] meanwhile the second is through a controlled aggregation[133, 134]. This explains that nucleation occurs throughout the reaction and the resulting nuclei (primary particles) will aggregate together to form larger particles (secondary particles).

Silica nanoparticles can be synthesized via Stöber process which involves hydrolysis and polycondensation of tetraethylorthosilicate under alkaline conditions in ethanol[135]: Bogush et al[133] successfully prepared monodispersed silica particles in the range of 40 nm to few micrometers using almost a similar method. The authors believe that the concentration of TEOS, ammonia, water, solvent (alcohol) and the reaction temperature, are the five key parameters, which govern the particle size and distribution.

By optimizing these parameters, Park et al. [136] conveniently prepared ultrafine silica particles within the range of 13.7 ± 4.5 nm.
Various authors have also shown how different levels of metal contaminants promote initiatives to produce purer particles with narrow size distribution. Kim et al. [137] were able to reduce the particle size up to 17.5 nm through addition of small amount of NaI during the synthesis. Rahman et al. [138, 139] reported that monodispersed silica of average size 20.5 ± 3.5 nm can be synthesized in the presence of small quantity of NH₄Br studying the effect of concentration of TEOS, ammonia and water, the feed rate of ammonia and reaction temperature on the resulting particle size and distributions of silica nanoparticles, under the influence of low frequency ultrasonic (42 kHz) and magnetic agitation.

Nanocomposite silica glassy layers (host) containing copper-based species (guest) were developed and tailored by the sol-gel route starting from ethanolic solutions of tetraethoxysilane (TEOS) and copper(II) acetate (Cu(CH₃COO)₂·4H₂O) in a single-step process and subsequently annealed ex situ under different atmospheres (air, nitrogen, or 4% H₂ in N₂ mixture) [140].

Another even more effective method for controlling the growth of the polymer and its final particle dimensions, also in the presence of NH₃, is by using nonionic inverse microemulsions. For such microemulsions, it is possible to obtain silica particles which are highly monodisperse and with diameters in the order of some tens of nanometers [141].

Polystyrene latex particles bearing basic groups on their surfaces were successfully synthesized through microemulsion polymerization using 4-vinylpyridine (4VP) as a functional comonomer and polyvinylpyrrolidone (PVP) as a surfactant [142]. A series of poly(styrene-co-4-vinylpyridine)/SiO₂ nanocomposite particles with smooth or rough core-shell morphology were obtained through the coating surface by reaction of TEOS in ethanol/water mixture.

Ahn et al [143] also reported a one-pot preparation of spherical silica microcapsules containing hydrophilic active compounds using a water-in-oil microemulsion process from the modified silicon alkoxides with 3-aminopropyl triethoxysilane (APTES) as a gelling agent. The H₂O to-TEOS molar ratio in the preparation of the encapsulation precursors played an important role on controlling the morphology and pore size of the microcapsules. The average particle sizes of the microcapsules were controllable in the
range of sub-micrometers to several tens of micrometers by adjusting agitation speeds, and the properties of the precursors. The pore size of the microcapsules can be also tuned by changing the H₂O-to-TEOS molar ratio in the preparation of the encapsulation precursors.

The inverse emulsion route to produce silica gel nanoparticles was used in the past also by our group\(^{144}\) for the sol–gel synthesis of silica nanoparticles under conditions that minimize denaturing effects on encapsulated enzymes. We have in particular focused on optimizing the purification procedures with the aim to produce water nanoparticles dispersions from water/oil emulsions without the use of precipitation/sedimentation steps at neutral pH. The process has been validated for enzyme encapsulation using horseradish peroxidase (HRP) as a model.

Another remarkable work reports a comparative study on structure-property relationship of silica hybrid nanocomposites \(^{145}\)(acrylic rubber (ACM)/silica, epoxidised natural rubber (ENR)/silica and poly (vinyl alcohol) (PVA)/silica) prepared by sol-gel technique under different pH levels (pH = 1.0-13.0): the pH of the solution, the mole ratio of Si to H₂O, catalysts, solvents and reaction temperature are key parameters that influence the sol-gel chemistry. Moreover, pH plays a key role in determining the nature of the hybrids when all other parameters are kept constant.

Landry et al.\(^{146}\) studied the effects of pH in poly (methylmethacrylate)/silica hybrids. The hybrids formed in both acidic and basic environments show that silica uniformly disperses in the polymer matrix with particles smaller than 100 nm using an acid catalyst, while these aggregate in basic medium. In preparing polymer-inorganic hybrids, HCl is commonly used as an acid catalyst. Huang et al.\(^{147}\) have observed the structure and morphology of the hybrids by using various HCl/TEOS ratios in poly (dimethyl siloxane).

Asaro et al.\(^{148}\) reported how the inverse microemulsion, comprehensively established for the synthesis of silica nanoparticles in an ammonia-catalyzed sol-gel process, can be alternatively studied with an acid-catalyzed sol-gel process.

Tetraethyl orthosilicate (TEOS) was used as the silica precursor, while two different aqueous phases containing either HNO₃ or HCl at two different concentrations, 0.1 and 0.05 M, were examined in the presence and in the absence of NaF, a catalyst of the condensation step.
Many authors have reported the synthesis of silica hybrid materials through basic or acidic catalyzed hydrolysis followed by in situ radical polymerization of various monomers: a hybrid material was obtained through basic catalysed sol–gel process of tetraethoxysilane (TEOS) and the alkoxysilyl unit of an hybrid monomer, followed by in situ free-radical polymerization to synthesize hybrid blends\textsuperscript{149-151}. The structure of the hybrid consisted of nanosilica, 10 nm in mean diameter, uniformly dispersed in the polymer phase (a hybrid monomer of 2-hydroxyethylmethacrylate (HEMA) and 3-Aminopropyltriethoxysilane (APTS)) with strong interactions between the phases. The obtained bioactive nanocomposite can be used to make bioactive scaffold for bone engineering.

Developing materials combining the advantages of synthetic polymers and bioactive glass nanoparticles by the sol gel process can provide efficient bone engineering scaffolds. In a recent study sol–gel bioactive glass (SG) nanoparticles were synthesized by quick alkali-mediated sol gel route \textsuperscript{152}. glass/poly(L-lactide) nanocomposite scaffolds were then developed, thus the effect of the glass content on the porosity and interconnectivity of the porous structure of the scaffold were studied. The polymer scaffold has a highly interconnected porous structure with a maximum pore size of about 250 μm. The degradation of composite scaffolds was also evaluated with a view to modulate degradation rate by increasing the glass content as well as in vitro bioactivity of the scaffolds by investigating the formation of the apatite layer on their surfaces during immersion in a simulated body fluid.

Another example of silica hybrid material was given by Ji \textsuperscript{153} with the synthesis of poly (ethylene terephthalate) (PET)/SiO\textsubscript{2} nanocomposites via the Sol–Gel method: terephthalic acid was first reacted with excess ethylene glycol to form bis (hydroxyethyl) terephthalate (BHET); then the tetraethoxysilane (TEOS) were transferred to the BHET followed by the Sol–Gel reactions at high temperature to form silica nanonetworks concurrent with polycondensation of BHET to produce the PET matrix.

Rosero-Navarro et al \textsuperscript{154} developed nanocomposite materials composed by silica nanoparticles in a hybrid organic–inorganic sol–gel matrix for corrosion protection of aluminium alloys. The sol–gel matrix was produced from an inorganic precursor,
tetraethoxysilane (TEOS), 3-metacryloxypropyltrimethoxysilane (MPS) and an organic bi-functional monomer, ethyleneglycol-dimethacrylate (EGDMA), used to develop a highly crosslinked organic network attached to the inorganic one through covalent Si–C bonds. Silica nanoparticles, on the other side, increase the density and provide a major mechanical performance through the reinforcement of the coating. The evolution of the sol, mainly the chemical structure, during the processes of hydrolytic condensation and organic polymerisation was studied as a function of the sol concentration.

A facile method for the synthesis of hollow polymer silica microspheres with different functional groups, such as amide, hydroxyl, and carboxyl, containing movable polyelectrolyte cores was presented by Ji et al. \[155]\: hollow polymer microspheres with movable quaternary pyridinium polyelectrolyte (PE) cores and various functional groups on the shell-layers, were prepared by the selectively etching of mid-silica layer with hydrofluoric acid from the corresponding poly(ethyleneglycol dimethacrylate-co-methacrylic acid, poly(ethyleneglycol dimethacrylate-co-4-vinylpyridinium benzylchloride)/silica/polymer (P(EGDMA-co-MAA) P(EGDMA-co-VPyBnCl)/SiO\(_2\)/polymer) tetra-layer microspheres. The tetra-layer hybrid microspheres were synthesized by a multistage reaction process, which included the combination of distillation precipitation polymerization for the formation of polymer-layers and the hydrolysis of tetraethyl orthosilicate (TEOS) via a modified Stöber sol–gel procedure to afford silica layer. The efficient electrostatic interaction between the cationic pyridinium species on the surface of P(EGDMA-co-MAA) P(EGDMA-co-VPyBnCl) cores and the negative charges on the silica species was essential to get monodisperse tri-layer P(EGDMA-co-MAA) P(EGDMA-co-VPyBnCl)/SiO\(_2\) microspheres during the hydrolysis of TEOS. The functional polymer shell was encapsulated over 3-(methacryloxy)propyl trimethacrylate (MPS) modified tri-layer polymer/silica seeds by distillation precipitation copolymerizations of N,N-methylenebisacrylamide (MBAAm) crosslinker and comonomers with different functional groups, including N-isopropylacrylamide (NIPAAm).

Through a modification of the Stöber process Siloxane-based nanoparticles have been successfully applied to ATRP systems to prepare various well-defined hybrid nanoparticles: the development of silica colloidal initiators, bearing benzyl chloride, 2-
bromopropionate, or 2-bromoisobutyrate groups, allowed the synthesis of hybrid colloidal nanoparticles by using the initiator groups in the ATRP of various vinyl monomers [61].

Zhao has reported on the ATRP of thermo-responsive polymer brushes on silica nanoparticles and the study of thermo-induced phase transitions in water: silica nanoparticles were specifically prepared by the Stöber process and the surface was functionalized by an ATRP initiator and then the surface-initiated ATRP of methoxy-di(ethylene glycol) methacrylate (DEGMMA) and methoxy-tri(ethylene glycol) methacrylate (TEGMMA) were carried out in THF at 40 °C in the presence of a free initiator, benzyl 2-bromoisobutyrate [96].

Silica nanoparticles of uniform size and precise morphology have been thus synthesized from the “sol-gel” approach [156], [157], [158] from the condensation of tetraethoxysilane (TEOS) [133],[136] achieving monodisperse SiO$_2$ particles; the size of the particles can be controlled by the initial concentrations of the reactants and the addition rate of TEOS; therefore the surface properties of silica colloids can be changed in a controllable way using siloxane chemistry to form self assemble monolayers (SAM) with the silanol groups [159].

1.5.4 Advantages of surface initiated ATRP onto nanoparticles

The ability to control molecular structure on atomic and macroscopic dimensions is a key parameter in designing materials with preprogrammed activity. A significant advance in this area has been the synthesis of nanocomposites where the structural order within the material can be controlled on nanometer/submicron scales.

The incorporation of well-defined organic and inorganic components into a singular material, in particular, the inclusion of well-defined polymers to inorganic substrates is of significance, because the functionality, composition, and dimensions of these macromolecules enable the design of specific properties into the resulting hybrid [160].

Recent developments in controlled/“living” radical polymerization (CRP) have provided a valuable methodology to introduce well-defined organic (co)polymers to a
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variety of inorganic substrates: hybrids containing nanoparticles have been prepared by other synthetic routes by trapping colloids within cross-linked matrixes, “grafting to” particles with functional molecules/polymers, or “grafting from” particles using a living or controlled polymerization process.

The properties of hybrid nanoparticles, prepared from controlled/“living” radical polymerization and particularly from ATRP, can be tuned by varying the particle size of the colloidal initiator, changing the composition of the particle core, or tethering (co)polymers with novel composition/functionality. An attractive feature of ATRP and other controlled/living radical processes is the ability to simultaneously grow chains from multifunctional cores or surfaces: specifically ATRP systems allow the facile functionalization of target substrates using commercially available α-haloesters, or benzyl halides, circumventing the multistep synthesis necessary for functional alkoxyamines and dithioesters [95]. Moreover, another interesting feature of hybrid nanoparticle ultrathin films has been the formation of ordered two-dimensional arrays of particles, with a spacing dependent on the radius of gyration of the tethered (co)polymer.

Ideally, in using efficient multifunctional initiators for ATRP, the number of functional initiating sites translates into stars or brushes possessing the exact number of tethered polymer chains. However, termination reactions at either low or high conversion may alter the functionality and molar mass distribution of tethered chains. An additional consideration in using multifunctional initiators is that termination can occur either by intermolecular coupling of growing radicals or in an intramolecular fashion by neighboring surface-immobilized chains. The impact of termination reactions on the synthesis of polymer brushes and stars is more significant than for linear polymers, as intermolecular termination results in crosslinking and intramolecular termination of immobilized polymeric radicals results in tethered chains possessing varying DP.

Another particularly interesting aspect of such spherical brushes is the possibility to achieve a high grafting density of polymer chains owing to the high curvature of the nanoparticle surfaces. In addition, the use of preformed, colloidal initiators opens the way to the systematic control of the brush length by changing the ratio of monomer to
initiating sites ([M]₀/[I]₀), while maintaining a core of static dimensions; furthermore, spherical brushes based on organic/inorganic hybrid materials serve as an interesting model to study the effect of confinement by densely grafting organic polymers onto an inorganic core.

1.6 Shear and dynamic rheology

Rheology is the study of the deformation and flow of matter. The rheological properties of a liquid are dominant features that can be quantified to characterize its behaviour and the response of a liquid to a forced shearing flow is the basis for determining the specific rheological properties of a given liquid. General qualitative terms used to describe these properties are viscoelastic, Newtonian, non-Newtonian, thixotropic and dilatant. Quantitative parameters used are viscosity, elasticity, shear rate, shear strain, and shear stress. The broadest view of liquid rheology is obtained by using oscillatory flow at a selected frequency because both viscous and elastic properties are revealed. Steady flow reveals only viscous properties. Values of shear stress, shear rate, and shear strain are primary parameters for quantitative specification of both the flow condition and the liquid response. It is from these quantities that the components of the viscoelastic modulus, the viscosity and the elasticity are obtained.

In addition to the quantitative specification above, it is useful to have a concept of the microstructure of a liquid, since that is the underlying physical basis for its rheological properties. A liquid with isotropic structure is one with perfectly random microstructure organization; in an anisotropic liquid the microstructure has a preferential directional orientation. The organization of the structural elements determines the way the liquid will flow, and microstructural organization is influenced by three distinct flow factors:

1. A liquid at rest (no flow) is isotropic, having no global preferential microstructural orientation.

2. Flowing liquid induces global anisotropic structure.

3. Flow induced anisotropy decays when flow is stopped.
Anisotropic particles (or macromolecules) in a liquid may collect together to form even larger anisotropic groups, but overall if their orientations are random, the liquid remains isotropic. Examples of anisotropic particles are bentonite plates, red blood cells, tobacco mosaic virus, biological macromolecules (hyaluronic acid, myosin, collagen, xanthan gum, dextran, etc.) and synthetic polymer chains.

**Figure 1-10.** Spherical sections of two types of suspended particles in a liquid. Both the rod type particles and the coiled particles are randomly oriented throughout the volume so that the suspensions are isotropic.

The shear forces due to flow cause an overall anisotropic reorganization of the microstructure of the liquid. The work done in producing the anisotropic global structure and accompanying flow is of two types: a recoverable energy associated with structure formation which is identified with the elasticity, and a lost energy dissipated in structural formation and sliding which is associated with the viscosity. Generally the anisotropy is increased with the rate of flow and accompanying increase of the shear forces.
Figure 1-11. Shear flow has the effect of applying tension and compression to the spherical section shown in figure 1-10. The result is a net alignment of the rods and a stretching and alignment of the coils so that the liquid now becomes anisotropic.

Neither the development nor loss of anisotropy is instantaneous because some finite time is required for the microstructure to change. The relaxation time is a measure of the rate at which the global structure changes in response to the change in flow. Thus, with changing flow the degree of anisotropy changes with the speed and time duration of the flow. When returning to the quiescent state (no flow), the liquid relaxes to the original global isotropic condition. The force of reorientation to the isotropic condition of rigid microstructural elements is due to Brownian motion, while shape recovery of flexible microstructural elements is aided by internal springs. The larger is the local structures, the longer is the relaxation time.
Figure 1-12. When the flow is suddenly stopped, the initial anisotropy begins decaying to the final isotropic limit. Correspondingly, the anisotropy will decrease in a manner determined by the type of suspended particles. Treating this decrease as an exponential function of time, an apparent relaxation time is defined as the time for the initial anisotropy to decrease by a factor of $1/e = 0.3678$.

When a viscoelastic liquid is subjected to oscillatory flow the anisotropic stress, strain and shear rate induce global anisotropy by rearrangement of the microstructure of the liquid. The anisotropy will vary during the flow cycle in an amount determined by the size of the period of oscillation and the relaxation time. With increasing amplitude of oscillatory flow, the anisotropic structure of the liquid is called on to store progressively increasing energy. But the ability of the flow-induced anisotropic microstructure to store energy is limited by its nature. As the shear strain exceeds unit value, the type of structure present undergoes a change which is identified by a maximum value of elastic stress (i.e. the maximum attainable energy stored per unit volume per unit strain). This is termed the "elastic yield stress".

While the microstructural relaxation time governs the structural response occurring on the time scale of the period of rapid changes in flow, some materials exhibit an additional change in microstructure that occurs over a much longer time scale, an effect called thixotropy.
Figure 1-13. The elastic yield stress is identified by a maximum in the elastic yield stress which occurs near a shear strain of 1. The maximum locates the elastic yield stress and the yield strain. Note that the stress is the energy per unit volume/unit strain, and the elastic yield stress is a maximum in the storage capacity of the elastic microstructure.

In oscillatory tests of thixotropic viscoelastic materials, changes in viscosity and elasticity appear over a period of time that is substantially longer than the period of oscillation. For example, when flow is suddenly initiated, the viscosity and elasticity change with time while the oscillatory flow is maintained constant. Similarly, reducing the flow yields viscoelasticity to change with time following the reduction. In some liquids these thixotropic effects also are seen when the viscosity and elasticity are measured for stepwise increasing amplitudes, then followed by decreasing amplitudes. In this situation the viscoelasticity is constantly trying to "catch up" with the flow condition and consequently the viscoelasticity for increasing flows differs from that for decreasing flows. Increasing flow usually degrades the microstructure while at the same time increasing anisotropy, although in some liquids flow can induce a microstructural enhancement giving rise to dilatancy. This property is identified by an anomalous increase in the viscosity or elasticity above the decrease expected with increasing flow.
amplitude. This condition occurs when the microstructure changes into a form that has an enhanced capacity for storage of elastic energy.

**Figure 1-14.** A thixotropic liquid exhibits a time dependent response to change in shear rate. Two types of time dependent flows are a suddenly initiated constant shear rate (left figure) and an incremental increase and decrease of shear rate (right figure). In both cases, the viscosity and elasticity change slowly with time.

Dilatancy is observed in measurement of the shear rate dependence of the viscosity and elasticity while holding the frequency of oscillatory flow constant.
Figure 1-15. Dilatancy is indicated by comparing the shear rate dependent changes in viscosity and elasticity (holding the frequency constant) of a liquid showing a normal response with a dilatant liquid which has an upturn tendency appearing at higher shear rates.

It is customary to represent the deformation behaviour of metals and other solids by a model called the linear or hookean elastic solid (displaying the property known as elasticity) and that of fluids by the model of the linear viscous or Newtonian fluid (displaying the property known as viscosity). These classical models are, however, inadequate to depict certain non-linear and time-dependent deformation behaviour that is sometimes observed. It is these nonclassical behaviours which are the chief interest of rheologists and hence referred to as rheological behaviour.

Rheological behaviour is particularly readily observed in materials containing polymer molecules which typically contain thousands of atoms per molecule, although such properties are also exhibited in some experiments on metals, glasses, and gases. Thus rheology is of interest not only to mathematicians and physicists, who consider it to be a part of continuum mechanics, but also to chemists and engineers who have to deal with these materials. It is of special importance in the plastics, rubber, film, and coatings industries.
Considering a block of material of height \( h \) deformed in the manner indicated in figure 1-16, the bottom surface is fixed and the top moves a distance \( w \) parallel to itself. A measure of the deformation is the shear strain \( \gamma \) given by the follow equation:

\[
\gamma = \frac{w}{h}
\]  

Eq. 1-14

**Figure 1-16.** Simple shear. (a) Undeformed block of height \( h \). (b) Deformed block after top has moved a distance \( w \) parallel to itself. The arrows indicate the net forces acting on the top and bottom faces. The forces which must be applied to left and right faces to maintain a steady state are not indicated.

To achieve such a deformation if the block is a linear elastic material, it is necessary to apply uniformly distributed tangential forces on the top and bottom of the block as shown in figure 1-16 (b). The intensity of these forces, that is, the magnitude of the net force per unit area, is called the shear stress \( S \). For a linear elastic material, \( \gamma \) is much less than unity and is related to \( S \) by the following equation:

\[
S = G \gamma
\]  

Eq. 1-17

where the proportionality constant \( G \) is a property of the material known as the shear modulus. If the material in the block is a Newtonian fluid and a similar set of forces is imposed, the result is a simple shearing flow, a deformation as pictured in figure 1-16 (b) with the top surface moving with a velocity \( dw/dt \). This type of motion is characterized by a rate of shear \( \dot{\gamma} = (dw/dt)/h \), which is proportional to the shear stress \( S \) as given by the following equation:

\[
S = \eta \dot{\gamma}
\]  

Eq. 1-18
where $\eta$ is a property of the material called the viscosity.

If the imposed forces are small enough, time-dependent deformation behaviour can often be described by the model of linear viscoelasticity. The material properties in this model are most easily specified in terms of simple experiments.

In a “creep” experiment a stress is suddenly applied and then held constant; the deformation is then followed as a function of time. This stress history is indicated in the solid line of figure 1-17 (a) for the case of an applied constant shear stress $S_0$. If such an experiment is performed on a linear elastic solid, the resultant deformation is indicated by the full line in figure 1-17 (b) and for the linear viscous fluid in figure 1-17 (c). In the case of elasticity, the result is an instantly achieved constant strain; in the case of the fluid, an instantly achieved constant rate of strain. In the case of viscoelastic materials, there are some which eventually attain a constant equilibrium strain (figure 1-17 (d)) and hence are called viscoelastic solids. Others eventually achieve constant rate of strain (figure 1-17 (e)) and are called viscoelastic fluids. If the material is linear viscoelastic, the deformation $\gamma(S_0, t)$ is a function of the time $t$ since the stress was applied and also a linear function of $S_0$ as reported in the following equation:

$$\gamma(S_0, t) = S_0 J(t)$$  \hspace{1cm} \text{Eq. 1-19}

where $J(t)$ is independent of $S_0$. The function $J(t)$ is a property of the material known as the shear creep compliance.
If stresses become too high, linear viscoelasticity is no longer an adequate model for materials which exhibit time-dependent behavior. In a creep experiment, for example, the ratio of the strain to stress, \( \gamma(t, S_0)/S_0 \), is no longer independent of \( S_0 \); this ratio generally decreases with increasing \( S_0 \). Two main examples of nonlinear viscoelasticity are shear thinning and thixotropy.
For polymer melts, solutions, and suspensions, generally speaking, the viscosity decreases as the shear rate increases. This type of behaviour, called shear thinning, is of considerable industrial significance. For example, paints are formulated to be shear-thinning. A high viscosity at low flow rates keeps the paint from dripping from the brush or roller and prevents sagging of the paint film newly applied to a vertical wall. The lower viscosity at the high deformation rates while brushing or rolling means that less energy is required, and hence the painter's arm does not become overly tired.

Thixotropy is a property of suspensions (for example, bentonite clay in water) which, after remaining at rest for a long time, act as solids; for example, they cannot be poured. However, if it is stirred, such a suspension can be poured quite freely. If the suspension is then allowed to rest, the viscosity increases with time and finally sets again. This whole process is reversible.

![Figure 1-18. Different types of responses to a change in strain rate.](image)

Depending on the change of strain rate versus stress inside a material the viscosity can be categorized as having a linear, non-linear, or plastic response. When a material exhibits a linear response it is categorized as a Newtonian fluid. In this case the stress is
linearly proportional to the strain rate. If the material exhibits a non-linear response to the strain rate, it is categorized as Non-Newtonian fluid. In addition, when the stress is independent of this strain rate, the material exhibits plastic deformation. Many viscoelastic materials exhibit rubber-like behaviour explained by the thermodynamic theory of polymer elasticity. In reality all materials deviate from Hooke's law in various ways, for example by exhibiting viscous-like as well as elastic characteristics. Viscoelastic materials are those for which the relationship between stress and strain depends on time. Anelastic solids represent a subset of viscoelastic materials: they have a unique equilibrium configuration and ultimately recover fully after removal of a transient load. All materials exhibit some viscoelastic response. In common metals such as steel or aluminium, as well as in quartz, at room temperature and at small strain, the behaviour does not deviate much from linear elasticity. Synthetic polymers, wood, and human tissue as well as metals at high temperature display significant viscoelastic effects.

Purely elastic materials do not dissipate energy (heat) when a load is applied, then removed. However, a viscoelastic substance loses energy when a load is applied, then removed. Hysteresis is observed in the stress-strain curve, with the area of the loop being equal to the energy lost during the loading cycle. Since viscosity is the resistance to thermally activated plastic deformation, a viscous material will lose energy through a loading cycle. Plastic deformation results in lost energy, which is uncharacteristic of a purely elastic material's reaction to a loading cycle.

Specifically, viscoelasticity is a molecular rearrangement. When a stress is applied to a viscoelastic material, such as polymer, part of the long polymer chain changes position. This movement or rearrangement is called creep. Polymers remain a solid material even when these parts of their chains are rearranging in order to accompany the stress, creating thus a back stress in the material. When the back stress is the same magnitude as the applied stress, the material no longer creeps. When the original stress is taken away, the accumulated back stresses will cause the polymer to return to its original form.
The red area is a hysteresis loop and shows the amount of energy lost (as heat) in a loading and unloading cycle. It is equal to:

\[ \int \sigma \, d\varepsilon \]

\text{eq. 1-20}

where \( \sigma \) is stress and \( \varepsilon \) is strain.

Linear viscoelasticity is when the function is separable in both creep response and load. All linear viscoelastic models can be represented by a Volterra equation connecting stress and strain:

\[ \varepsilon(t) = \frac{\sigma(t)}{E_{\text{inst,creep}}} + \int_0^t K(t - t') \sigma'(t') \, dt' \]

\text{eq. 1-21}

Or

\[ \sigma(t) = E_{\text{inst,relax}} \varepsilon(t) + \int_0^t F(t - t') \varepsilon'(t') \, dt' \]

\text{eq. 1-22}
Where $t$ is time $\sigma(t)$ is stress, $(t)$ is strain, $E_{\text{inst,creep}}$ and $E_{\text{inst,relax}}$ are instantaneous elastic moduli for creep and relaxation, $K(t)$ is the creep function and $F(t)$ is the relaxation function; linear viscoelasticity is usually applicable only for small deformations.

Nonlinear viscoelasticity is when the function is not separable. It usually happens when the deformations are large or if the material changes its properties under deformations.

### 1.6.1 Dynamic modulus

Viscoelasticity is studied using dynamic mechanical analysis applying a small oscillatory strain and measure the resulting stress.

Purely elastic materials have stress and strain in phase, so that the response of one caused by the other is immediate. In purely viscous materials, strain lags stress by a 90 degree phase lag.

Viscoelastic materials exhibit behaviour somewhere in the middle of these two types of material, exhibiting some lag in strain.

Complex Dynamic modulus $G$ can be used to represent the relations between the oscillating stress and strain:

$$G = G' + i G''$$  \hspace{1cm} \text{eq. 1-23}

where $\dot{r} = -1$, $G'$ is the storage modulus and $G''$ is the loss modulus:

$$G' = \frac{\sigma_0}{\varepsilon_0} \cos \delta$$  \hspace{1cm} \text{eq. 1-24}

$$G'' = \frac{\sigma_0}{\varepsilon_0} \sin \delta$$  \hspace{1cm} \text{eq. 1-25}

where \( \sigma_0 \) and \( \varepsilon_0 \) are the amplitudes of stress and strain and $\delta$ is the phase shift between them.
Viscoelastic materials, such as amorphous polymers, semicrystalline polymers, and biopolymers, can be modelled in order to determine their stress or strain interactions as well as their temporal dependencies. These models, which include the Maxwell model, the Kelvin-Voight model and the Standard Linear Solid model, are used to predict a material response under different loading conditions. Viscoelastic behaviour has elastic and viscous components modelled as linear combinations of springs and dashpots, respectively. Each model differs in the arrangement of these elements, and all of these viscoelastic models can be equivalently modelled as electrical circuits. In an equivalent electrical circuit, stress is represented by voltage and the derivative of strain (velocity) by current. The elastic modulus of a spring is analogous to a circuit's capacitance (it stores energy) and the viscosity of a dashpot to a circuit's resistance (it dissipates energy). The elastic components, as previously mentioned, can be modelled as springs of elastic constant \( E \), given the formula:

\[
\sigma = E \varepsilon
\]

\[\text{eq. 1-26}\]

where \( \sigma \) is the stress, \( E \) is the elastic modulus of the material, and \( \varepsilon \) is the strain that occurs under the given stress, similar to Hooke’s Law.

The viscous components can be modelled as dashpots such that the stress-strain rate relationship can be given as:

\[
\sigma = \eta \frac{d\varepsilon}{dt}
\]

\[\text{eq. 1-27}\]

where \( \sigma \) is the stress, \( \eta \) is the viscosity of the material, and \( d\varepsilon/dt \) is the time derivative of strain.

The relationship between stress and strain can be simplified for specific stress rates. For high stress states/short time periods, the time derivative components of the stress-strain relationship dominate. A dashpot resists changes in length and in a high stress state it can be approximated as a rigid rod. Since a rigid rod cannot be stretched past its original length, no strain is added to the system.
Conversely, for low stress states/longer time periods, the time derivative components are negligible and the dashpot can be effectively removed from the system, an "open" circuit. As a result, only the spring connected in parallel to the dashpot will contribute to the total strain in the system.

The Maxwell model can be represented by a purely viscous damper and a purely elastic spring connected in series, as shown in figure 1-20. The model can be represented by the following equation:

$$\frac{d\varepsilon_{Total}}{dt} = \frac{d\varepsilon_D}{dt} + \frac{d\varepsilon_S}{dt} = \frac{\sigma}{\eta} + \frac{1}{E} \frac{d\sigma}{dt}$$

Under this model, if the material is put under a constant strain, the stresses gradually relax; when a material is put under a constant stress, the strain has two components. First, an elastic component occurs instantaneously, corresponding to the spring, relaxing immediately upon release of the stress. The second is a viscous component that grows with time as long as the stress is applied. The Maxwell model predicts that stress decays exponentially with time, which is accurate for most polymers. One limitation of this model is that it does not predict creep accurately. The Maxwell model for creep or constant-stress conditions postulates that strain will increase linearly with time. However, polymers for the most part show the strain rate to be decreasing with time.

![Figure 1-20](image) Schematic representation of Maxwell model.

The Kelvin–Voigt model, also known as the Voigt model, consists of a Newtonian
damper and Hookean elastic spring connected in parallel, as shown in figure 1-21. It is used to explain the creep behaviour of polymers.

The constitutive relation is expressed as a linear first-order differential equation:

\[ \sigma(t) = E \varepsilon(t) + \eta \frac{d\varepsilon(t)}{dt} \]

\[ \text{eq. 1-29} \]

This model represents a solid undergoing reversible, viscoelastic strain. Upon application of a constant stress, the material deforms at a decreasing rate, asymptotically approaching the steady-state strain. When the stress is released, the material gradually relaxes to its undeformed state. At constant stress (creep), the model is quite realistic as it predicts strain to tend to \( \sigma/E \) as time continues to infinity. Similar to the Maxwell model, the Kelvin–Voigt model also has limitations. The model is extremely good with modelling creep in materials, but with regards to relaxation the model is much less accurate.

![Figure 1-21. Schematic representation of Kelvin–Voigt model.](image)

A third model, called the Standard Linear Solid, effectively combines the Maxwell model and a Hookean spring in parallel. A viscous material is modelled as a spring and a dashpot in series with each other, both of which are in parallel with a lone spring
For this model, the governing constitutive relation is:

\[
\frac{d\varepsilon}{dt} = \frac{E_2}{\eta} \left( \frac{\eta}{E_2} \frac{d\sigma}{dt} + \sigma - E_1 \varepsilon \right) \frac{E_1 + E_2}{E_1 + E_2}
\]

\[eq. \ 1-30\]

Under a constant stress, the modelled material will instantaneously deform to some strain, which is the elastic portion of the strain, and after that it will continue to deform and asymptotically approach a steady-state strain. This last portion is the viscous part of the strain. Although the Standard Linear Solid model is more accurate than the Maxwell and Kelvin-Voigt models in predicting material responses, mathematically it gives inaccurate results for strain under specific loading conditions and is rather difficult to calculate.

Figure 1-22. Schematic representation of the Standard Linear Solid model.
1.7 Scope of the thesis

Many efforts had been made in the last few years to control radical polymerizations in order to obtain macromolecules with predetermined molecular weight and low polydispersity and to synthesize complex architecture copolymers (block, graft, star) and several controlled / “living” radical polymerization techniques had been developed and their potential explored.

This project reports on new products and processes derived from the use of so called “living” radical polymerizations, specifically ATRP, that allows the preparation of well-defined macromolecular structures.

A procedure for coating surfaces with polymer layers was established. Atom transfer radical polymerization was used for polymerizing water-soluble monomers onto silica nanoparticles, intended as a model for any polymeric functional surface. Silica nanoparticles, have shown promise in a wide variety of biomedical applications, where they are generally used for medical diagnosis and as nanocarriers for drug delivery and enzyme encapsulation. For an in vivo administration, however, nanoparticles should be protein repellent in order to avoid recognition and clearance by phagocytic cells. This “stealth character” can be achieved by decorating their surface with hydrophilic and protein-repellent polymers, which can be eventually functionalized to obtain more sophisticated biologically responsive nanoparticles.

The scope of this work is thus the study of the waterborne surface-ATRP scheme for the nano-sized spherical brushes synthesis: specifically, the synthesis of a cationic ATRP macroinitiator, which can be adsorbed on the anionic surface of silica nanoparticles, and the subsequent growth of hydrophilic polymers via ATRP onto colloidal silica particles will be studied in order to achieve nanostructured materials possessing a stealth character and functionality.
1.8 References

Chapter 2

2 Functionalization of silica nanoparticles

2.1 Summary

Atom transfer radical polymerization (ATRP) offers a number of attractive features, among them, the ability to simultaneously grow macromolecules from multifunctional cores (Scheme 2-1) by direct functionalization of ATRP initiator groups onto nanosized colloids and subsequent surface-initiated growth of polymer brushes.

In this section we have applied ATRP to the development of bio-hybrids nanoparticles, which are characterized by the presence of encapsulated, but active enzymes. We have here synthesized ATRP macroinitiators and studied their adsorption on model silica systems; we have then monitored the kinetics of surface-initiated polymerization and finally applied this technology to enzyme-containing nanoparticles.

![Scheme 2-1. Representative scheme of the synthesis of core-shell silica brushes using colloidal silica nanoparticles by a) adsorption of polymers bearing ATRP initiators groups (X) and b) subsequent grafting of functional macromolecules by atom transfer radical polymerization.](image)

Naked silica  |  Colloidal SiO$_2$ macroinitiators  |  Core-shell silica brushes

SiO$_2$  |  SiO$_2$  |  SiO$_2$
2.2 Introduction

We have focused on the development of silica nanostructured systems: our group has demonstrated that enzymes can be encapsulated in an active form in \textit{in situ} prepared silica gel nanoparticles \cite{1} while the surface of these nanoparticles can be easily functionalized, e.g. using atom transfer radical polymerization to grow polymer brushes from their surfaces.

The synthesis of brushes on particle surfaces has been widely studied to prepare solid supports \cite{2,3}, chromatographic stationary phases \cite{4-7} or high surface area models for brushes on flat surfaces \cite{8-11}; brushes were grown by surface-initiated ATRP, from organic latex colloids emulsions \cite{12-18}, shells of shell-cross linked micelles \cite{19,20} and from functionalized inorganic particles, such as silica \cite{3,4,7-10,21-33}, gold \cite{34,35}, alumina \cite{36}, polysilsesquioxane \cite{37}, titanium oxide clusters \cite{38,39}, iron oxides \cite{40} and germanium \cite{41}.

Herein we specifically focus on the aqueous ATRP synthesis of colloidal brushes by functionalizing silica nanoparticles with polyglycerol monomethacrylate.

2.2.1 Aqueous ATRP

During the past few years much effort has been devoted to the use of living/controlled radical polymerization in a water environment. Water is cheap, nontoxic, promotes rapid polymerization, may also facilitate removal of catalyst residues and these environmentally friendly conditions are expected to be particularly attractive for the eventual commercial exploitation of ATRP. Nonetheless, the number of water-soluble monomers polymerized in controlled fashion is still limited and side reactions often hamper the preparation of high molecular weight polymers.

Matyjaszewski and co-workers \cite{42} have reported in 1997 one of the first example of well controlled (co)polymerization of acrylates in water medium: the controlled/“living” polymerization of a functional monomer, 2-hydroxyethyl acrylate, using halogenated initiators and CuBr/ bipyridine as catalyst, in 1 : 1 (by volume)
aqueous solution at 90 °C was successfully achieved: the reaction exhibited first-order kinetic, molecular weights increased linearly with conversion and polydispersities remained low (M_w/M_n = 1.2) throughout the polymerization, demonstrating thus the resilience of ATRP to protic media.

An example of the atom transfer radical polymerisation of monomers in aqueous media was described by Armes and co-workers \[^{43}\] by the synthesis of methacrylic copolymers: sodium methacrylate was polymerised directly via ATRP using a poly(ethylene oxide)-based macro-initiator achieving copolymers with narrow molecular weight distributions; Armes et al. \[^{44}\] efficiently synthesized another basic monomer, sodium 4-vinylbenzoate (NaVBA), via ATRP in aqueous media at ambient temperature: in contrast to sodium methacrylate, excellent yields were obtained in very short reaction times even at 20 °C.

Further proof of the successful use of water as reaction medium was the synthesis of another hydrophilic monomer, methoxy-capped oligo(ethylene glycol) methacrylate (OEGMA), described by Wang et al. \[^{45,46}\]; the use of various bromide-based initiators, in conjunction with a copper-based catalyst and two ligands, namely 2,2’-bipyridine (bpy) and 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) allowed rapid homopolymerization of OEGMA in mild conditions (water at 20 °C), achieving a good living character; indeed, high conversions (90%) were achieved within 20 min, molecular weight increases linearly with conversion and polydispersities resulted fairly narrow (M_w/M_n = 1.15-1.30) throughout the polymerization.

Moreover it was remarkably highlighted the highly active nature of the copper catalyst in water as it was possible to lower its amount by a factor of 10 (i.e., [Cu(I)]/[initiator] = 0.10) without significant loss of control over the polymerization, easing thus its removal from the aqueous reaction solution \[^{47}\].

Perrier and co-workers \[^{47}\] reported a detailed investigation on the copper(I)-mediated living radical polymerization of methoxypolyethylene oxide methacrylate using N-(n-alkyl)-2-pyridylmethanimine ligands in aqueous solution and the influence of temperature, [Cu(I)], and [Cu(II)] on the rate of polymerization. When the catalyst concentration was reduced by two orders of magnitude, the rate of polymerization was reduced with 100% conversion achieved with the M_n of the final product being higher than that predicted and the polydispersity equal to 1.43; in order to circumvent this
problem copper(II) was added as inhibitor, thus replacing 90% of Cu(I) by Cu(II) the rate of polymerization decreased significantly and narrow polydispersity (M_w/M_n = 1.15) was achieved. Optimum results were obtained by also increasing the amount of catalyst, with 100% of conversion and M_w/M_n = 1.15.

Thus, water was demonstrated to be an effective polymerization medium to achieve polymers with controlled number-average molecular masses and narrow polydispersities optimizing a range of options, as reducing the level of catalyst and the addition of inhibitors.

However, the nature of the solvent used for polymerization plays a crucial role in the activity of the catalyst, thus the choice of the medium has to be carefully considered in each case.

Masci and co-workers have described the advantages of the use of a DMF/water mixed solvent in the synthesis of new amphiphilic, ionic and thermo-responsive block copolymers of methacrylates \cite{48,49} and acrylamides \cite{50,51} without the use of protecting group chemistry: the first example of well-controlled atom transfer radical polymerization (ATRP) of a permanently charged anionic acrylamide monomer, sodium 2-acrylamido-2-methylpropanesulfonate (NaAMPS) \cite{51} was achieved with ethyl 2-chloropropionate (ECP) as an initiator and the CuCl/CuCl_2/tris(2-dimethylaminoethyl) amine (Me_6TREN) catalytic system.

The polymerization was carried out in 50:50 (v/v) N,N-dimethylformamide (DMF)/water mixtures at 20°C. Linear first-order kinetic plots up to 92% conversion for a target degree of polymerization of 50 were obtained, molecular weight increased linearly with the conversion in good agreement with the theoretical values and the polydispersities decreased with increasing conversion, down to M_w/M_n = 1.11. Moreover the living character of the polymerization was further confirmed by chain-extension experiments and synthesis of block copolymers with N,N-dimethylacrylamide and N-isopropylacrylamide.

Controlled polymerization of the thermo-responsive monomer N-isopropylacrylamide (NIPAAm) \cite{50} was also achieved by atom transfer radical polymerization (ATRP) in DMF:water 50:50 (v/v) mixed solvent at 20 °C using similar reaction conditions and block copolymers with N,N dimethylacrylamide (DMAAM) and 3-sulfopropyl methacrylate (SPMA) were successfully prepared.
The synthesis of potassium 3-sulfopropyl methacrylate (SPMA) homopolymers and amphiphilic block copolymers with methyl methacrylate (MMA) by ATRP in water/DMF mixed solvent was also investigated by Masci et al. \[48\] using Cu/bipyridine catalyst and halogen exchange. In water:DMF 50:50 (v/v), addition of at least 60% of Cu(II)Cl\(_2\) with respect to Cu(I)Cl allowed to obtain a good control of the polymerization, linear kinetic plots up to very high conversion were observed and the final polydispersities were relatively low (\(M_w/M_n = 1.15-1.25\)).

Amphiphilic block copolymers with MMA were then directly prepared in water:DMF 40:60 (v/v) without using protecting group chemistry or post-polymerization derivatization, with linear first-order kinetic plot for MMA polymerization up to 96% conversion; the presence of at least 60% of Cu(II) with respect to Cu(I) was required in order to achieve a good control over the polymerization.

The versatility of water/organic solvent ATRP was further put in evidence by the grafting of polysaccharides with methacrylate and acrylamide monomers in homogeneous mild conditions \[49\]: various compositions of water/DMF mixtures were used as solvent for the “grafting-from” of pullulan and dextran ATRP macroinitiators with a well controlled degree of functionalization, obtaining good control over the number, molecular weight and polydispersity of the grafted chains without homopolymer formation.

The versatility of this method was thus demonstrated by preparing ionic, amphiphilic, and thermoresponsive derivatives: in the latter case, the control on molecular weight and polydispersity allowed to introduce an important additional control over the LCST.

As a general result, it has emerged that ATRP in aqueous homogeneous media is fast but it can produce polymers of relatively high polydispersity index, indicating loss of control \[52, 53\]; however, the accelerating effect of water has been highlighted also in mixed organic/water environment \[44, 45, 52\]. This effect was mainly attributed to the displacement of the nitrogen ligands from the coordination sphere of the copper complex by water molecules (aquation) or hydroxyl ions (hydrolysis) \[54-59\]; this exchange would reduce the deactivator concentration and therefore lower the rate of deactivation. However also polarity effects were also credited as being a possible cause for the fast ATRP reactions in the presence of water \[44, 45, 51\]; furthermore the
“aquated” complexes of the transition metal at their higher oxidation state may cause termination by the outer sphere electron-transfer process \[60,61\].

A recent study on the ATRP deactivation efficiency and degree of control reported by Tsarevsky et al. \[62\] specifically focuses on the role of water and protic solvents: several side reactions in ATRP systems in presence of water, such as hydrolytic displacement of the halogen atom from the initiator or from the dormant species, especially at elevated temperatures, disproportionation of the Cu(I)-based ATRP catalyst and hydrolysis of the ATRP deactivator \[53\] may occur eventually leading to fast polymerizations and loss of control; however the solvent can, in principle, affect \(k_p\), \(K_{ATRP}\), and the deactivator concentration \([\text{Cu}^{II}L_nX]\) too: the influence of water or other protic solvents on the activity of a polar monomer (thus on the \(K_p\)) is usually related to the strength of hydrogen bonds but the increase in polymerization rate was attributed to either an increase of \(K_{ATRP}\) or a decrease of the deactivator concentration in presence of water; if the deactivator concentration in the reaction system is depleted by the halide ligand, dissociation and/or competitive complexation with solvent, as presented at the bottom of Scheme 2-2, then the deactivation will be slower. In summary, the equilibrium constant \(K_{ATRP}\) as well as the deactivator concentration \([\text{Cu}^{II}L_nX]\), which determine the ATRP rate and the degree of control over polymerization, depend on the reaction medium composition.

![Scheme 2-2](image-url)

**Scheme 2-2.** Basic equilibria in a generic aqueous ATRP system: the reversible dissociation of deactivator \(\text{Cu}^{II}L_nX\) may bring to complexation of the species \(\text{Cu}^{II}L_n\) or \(X\) with water (aquation).
2.2.2 Choice of the monomer

2,3-Dihydroxypropyl methacrylate (Figure 2-1), also known as glycerol monomethacrylate (GMMA), is a highly hydrophilic monomer of commercial interest; hydrogels based in GMMA have already been studied for some years. Due to its increased hydrophilicity, GMMA is a candidate for replacing the less hydrophilic 2-hydroxyethyl methacrylate (HEMA) in products such as soft contact lenses, hydrogels, drug delivery, and other medical applications. Furthermore, it has been investigated as material for ultrafiltration barriers mimicking the behavior of natural membranes in kidneys.

![Figure 2-1. Chemical structure of 2,3-Dihydroxypropyl methacrylate (glycerol monomethacrylate).](image)

Although poly(glycerol monomethacrylate) (PGMMA) homopolymer and copolymers with styrenes, isoprene and some methacrylates have been synthesized before by anionic polymerization, GMMA monomer has recently been directly homo- and copolymerized by ATRP.

Save and co-workers reported on the homopolymerization of glycerol monomethacrylate (GMA), using ATRP chemistry in aqueous, methanolic, or water/methanol solution: in methanol, monomer was polymerized to high conversion with reasonably good control and low polydispersity (Mw/Mn = 1.30) at 20 °C and “self-blocking” chain growth experiments indicated good living character; however the addition of water leads to much more rapid polymerizations but with high polydispersity (e.g. Mw/Mn = 1.90 for a 50/50 water/methanol mixture).

Topham et al. described the synthesis of well-defined glycerol monomethacrylate macromonomer by the judicious combination of atom transfer radical polymerization (ATRP) and copper-catalyzed 1,3-dipolar cycloaddition (azide-alkyne click chemistry). An azido R functionalized ATRP initiator was used to produce well-defined
homopolymers with terminal azide functionality via ATRP in protic media at 20 °C, with generally good control being achieved over both target molecular weight and final polydispersity ($M_w/M_n = 1.10-1.35$).

The synthesis of near-monodisperse acidic homopolymers and block copolymers was achieved using hydroxylated methacrylic copolymers esterified: hydroxylated polymer were synthesized via ATRP of glycerol monomethacrylate (GMMA) and then esterified using excess acid anhydride under mild conditions. Various novel diblock and triblock copolymers containing GMMA units with amphiphilic, pH and thermoresponsive behavior were achieved: Jiang et al. synthesized a triblock copolymer, poly(ethylene glycol)-b-poly(glycerol monomethacrylate)-b-poly(2-(diethylamino)ethyl methacrylate) (PEG-PGMA-PDEA), via atom transfer radical polymerization (ATRP) using a PEG-based macroinitiator for the preparation of shell-core micelles with pH-induced behaviour; another triblock copolymer, poly(glycerol monomethacrylate)-b-poly(2-(dimethylamino)ethyl methacrylate)-b-poly(2-(diethylamino)ethyl methacrylate) (GMA-DMA-DEA) was synthesized by via ATRP to investigate specifically the pH-induced micellization kinetics.

Amphiphilic water soluble triblock copolymers based on poly(glycerol monomethacrylate) and thermoresponsive poly(propylene oxide) were successfully synthesized via the ATRP technique: unimodal molar mass distributions and relatively low polydispersities were obtained for different lengths of the PGMA block and their association behaviour in aqueous solutions was studied in order to evaluate the micelle dimensions and the influence of temperature on micellar size.

Furthermore, Edmondson and co-workers reported an example on the use of glycerol monomethacrylate as a precursor of a polyelectrolytic macroinitiator for surface initiated polymerization: the one-pot synthesis of a new anionic macroinitiator based on esterification of poly(glycerol monomethacrylate) with 2-bromoisobutyryl bromide, followed by excess 2-sulfobenzoic acid cyclic anhydride and its electrostatic adsorption onto anaminated (cationic) planar substrate were efficiently prepared for the surface-initiated ATRP of various hydrophilic methacrylic monomers in alcohol/water solvent mixtures.
Double hydrophilic poly(ethylene oxide)-b-poly(glycerol monomethacrylate)-(PEO-b-PGMMMA) copolymers synthesized by atom transfer radical polymerization (ATRP), using a PEO-based macroinitiator, were used for post-polymerization conjugation (via Steglich esterification) to an hydrophobic nonsteroidal anti-inflammatory agent indomethacin (IND) \(^{[78]}\): the resulting amphiphilic macromolecule-drug conjugates (PEO-b-(PG2MA-IND)) were found to self-assemble into spherical micellar nanoparticles or vesicles in selective solvent (water), therefore originating a particular core-shell system in which the core-forming block (PG2MA-IND) resembled the drug to be physically encapsulated (IND). Hence, such micellar nanocontainers were able to transport two or more hydrophobic molecules, IND chemically linked to the polymer via acid sensitive bonds and another physically entrapped, which can be released either simultaneously in a pH-triggered process or selectively by initial passive diffusion of unbound species.

Among other important biomedical applications, Iddon and Armes \(^{[79]}\) described the successful synthesis of new stimulus-responsive block copolymer gelators based on GMMA: bifunctional and trifunctional ATRP initiators were used in 2-propanol/water mixtures at 20 °C to prepare a triblock and a three-arm star diblock copolymers, in which the central block comprised poly(glycerol monomethacrylate) and the outer blocks comprised of pH-responsive poly(2-(diethylamino)ethyl methacrylate) or poly(2-(diisopropylamino) ethyl methacrylate); the resulting copolymers formed hydrogels at neutral pH on addition of a base.

Targeted or time-delayed drug delivery have been nowadays the main focus of research in the area of stimulus-responsive gels: such gels that release biomedically active compounds locally under specific physiological circumstances or allow sustained release over long time periods are highly desirable \(^{[80]}\) and the controlled release systems can improve the efficacy of drug therapy, reducing the need for specialised or repeated drug administration and also minimising side effects \(^{[81]}\).

A further example of the use of GMMA for potential applications in enzyme catalysis and biosensors was provided by Huang and co-workers \(^{[82]}\): poly[(glycidyl methacrylate)-co-(glycerol monomethacrylate)]-grafted magnetic microspheres were prepared by graft random copolymerization via ATRP from polymer microspheres containing dispersed Fe\(_3\)O\(_4\) nanoparticles; Penicillin G acylase (PGA) was immobilized
onto the polymer brush-grafted spheres; the poly(glycidyl methacrylate) units, which acted as functional groups, were able to directly form stable covalent linkages with penicillin G acylase under relatively mild experimental conditions. On the other hand, the hydrophilic poly(glycerol monomethacrylate) units enabled the microspheres to disperse well in an aqueous environment. The immobilized PGA spheres showed high thermal stability and enhanced tolerability to the pH variance; furthermore, hydrophilicity and flexibility of the grafting chains efficiently reduce diffusion limitation thus rendering these systems good carriers for enzyme immobilization as industrial biocatalysts.

Due to the numerous examples of the use of pGMMA for bio-applications, its use for grafting silica nanoparticles could be quite promising in the development of clinically relevant bio-nanomaterials.

### 2.3 Experimental Section

#### 2.3.1 Materials

Ethyl α-bromoisobutyrate (EBiB, Aldrich, 98%), triethylamine (TEA, Aldrich, 98%), α-bromoisobutyryl bromide (BriBuBr, Aldrich, 98%), methyl iodide (CH$_3$I, Aldrich, 98%), 2,2’-bipyridyl (bpy, Aldrich, 98%), glycerol monomethacrylate (GMMA, Cognis Performance Chemicals, Hythe, UK), hydrochloric acid (Fluka, 1 M), sodium hydroxide (Fluka, 1 M), iodomethane (Sigma-Aldrich, 99%), methanol (Fluka, HPLC grade), acetonitrile HPLC grade (BDH), tetrahydrofuran (ACS reagent, ≥99.0%), N,N-dimethylformamide (Fuka) (hexane (ACS reagent, Aldrich, ≥95%) and dichloromethane (DCM, Sigma-Aldrich, ≥99.5%), LUDOX® TMA colloidal silica sol with a nominal mean particle diameter of 20 nm (Aldrich, 34 % wt. suspension in H$_2$O), silica nanoparticles (SiO$_2$-R-L1285, 5% wt., av. diameter 235 nm, nanoparticles GmbH), tetraethyl orthosilicate (TEOS, Aldrich, 98%), Silica 60, 0.04-0.063 mm/230-400 mesh and Celite (Macherey-Nagel), peroxidase from horseradish (HRP, Sigma, 150-250 units/mg solid), phosphate buffered saline (PBS, Sigma, pH=7.4) were used as received.
Copper (I) bromide (CuBr, Aldrich, 98%), copper (I) chloride (CuCl, Aldrich, ≥99%), copper (II) chloride (CuCl₂, Aldrich, 97%) were used as received without further purification but always keeping them under a protective argon atmosphere. 2-(N,N-dimethylamino)ethyl methacrylate (DMAEMA, Aldrich, 98% and 2-Hydroxyethyl methacrylate (HEMA, Aldrich, 98%) were freshly distilled under reduced pressure prior to polymerization.

2.3.2 Physico-chemical characterization

**UV-Vis** (enzymatic assays). A temperature-controlled UV-Vis Lambda 12 spectrometer (Perkin Elmer) was used to monitor the optical density at 420 nm at a temperature of 20°C. Typically, 0.1 mL of PBS containing variable amounts of HRP (0.4 - 0.7 unit/mL of peroxidase in the calibration) was mixed with 0.16 mL of hydrogen peroxide solution (0.50 % wt.) and 0.32 mL of a 5% wt. pyrogallol solution in 2.42 mL of 100 mM PBS (pH 6.0). The experimental procedure of the enzymatic assay follows the Sigma Aldrich procedure Enzymatic Assay of Peroxidase (EC 1.11.1.7).

**ATR-IR.** Infrared spectra were recorded using a Tensor IR Bruker and the ATR spectra were elaborated using Opus software.

**1H-NMR.** NMR spectra were recorded using a Bruker AVC spectrometer operating at 300 MHz utilizing the XwinNMR 3.5 software for the subsequent elaboration. 10 mg of sample were dissolved in 1 mL of deuterated chloroform or DMSO.

**HPLC.** An HPLC system based on a 250 * 4.0 mm Laserchrom C₁₈ Water column, on a UV/VIS detector 3210 (Laserchrom) operating at 230 nm nm and on a Clarity Chromatography software was used to evaluate monomer consumption. For the analysis, 10 μL of the reaction mixture were diluted in 6 mL of water: CH₃CN (90:10 v/v) and filtered through a 0.45 μm filter; 20 μL were finally injected. The monomer concentration in each sample was obtained normalizing the area of the monomer peak by the area of DMF (used as the internal standard). The kinetics of the reaction were
studied plotting the logarithm of the ratio between initial monomer concentration and monomer concentration at time t as a function of time.

**DLS and ζ-potential.** The measurements were performed by Zetasizer nanoseries ZEN3600 (Malvern Instruments) equipped with a solid state laser (λ = 633 nm).

**Thermogravimetric analysis (TGA).** Freeze dried samples (weight ranging from 1 mg to 10 mg) were analyzed with a Q5000IR TGA (TA Instruments, Delaware USA). Each measurement was performed under nitrogen flow (25 mL/min), using a 60 minutes initial isotherm at 120°C and then heating at a speed of 10°C/min.

**Atomic Force Microscopy (AFM).** 20 μL of sample solution were placed on a freshly cleaved mica substrate until complete evaporation. All the measurements were performed at room temperature using a Molecular Force Probe 3D AFM (MFP-3D, Asylum Research, Santa Barbara, CA) equipped with a 90 μm scanner. Silicon cantilevers (model AC-240, Asylum Research) with a nominal spring constant of 2 N/m and a resonance frequency of 70 kHz were utilized in all measurements. The apparatus was allowed to equilibrate for 15 minutes prior to each measurement to reduce the thermal drift and afterwards images were collected using the tapping mode imaging and a scan rate of 1 Hz.

### 2.3.3 Synthesis of macroinitiators

We have synthesized macroinitiators with 2 different compositions (DMAEMA:HEMA molar ratio: 1:1 and 3:1) and 3 degrees of polymerization, which were controlled through the monomer/initiator ratios. The monomer ratio, the degree of esterification (the percentage of isobutyryl groups with respect to the HEMA units) and the degree of quaternisation were calculated from the 1H-NMR analysis of the copolymers.

**First step.** For a 3:1 DMAEMA:HEMA ratio, 7.85 g (49.9 mmol) of DMAEMA and 2.17 g (16.6 mmol) of HEMA (16.5 mmol) were introduced in three different Schlenck tubes, where three different amounts of ethyl 2-bromo isobutyrate, 162 mg (0.832
mmol), 271 mg (1.39 mmol) and 540 mg (2.77 mmol) respectively, were then introduced. Each tube was placed in a thermostatic bath at 20°C and purged with nitrogen and subjected to three vacuum-nitrogen cycles to remove traces of oxygen. 10 mL of previously degassed methanol, then CuBr (119 mg, 199 mg and 397 mg respectively) and 2,2'-bipyridyl (260 mg, 434 mg, 865 mg respectively) were quickly introduced under nitrogen and the polymerization was then kept at 20°C for 16 hours; the dark brown colour was maintained throughout the polymerization ensuring thus a negligible oxidation of Cu(I) by ingress of oxygen. The solutions were then precipitated in hexane, the solid was redissolved in dichloromethane, precipitated two additional times in hexane, and finally filtered through a silica column (silicagel 60) using dichloromethane as an eluent to remove catalyst traces, and finally dried by rotary evaporation. Typical yield (relative to a quantitative conversion of the monomer mixtures): 90%.

1H NMR (CDCl₃): δ = 0.84-0.99 (α, CH₃ in pDMAEMA and pHEMA main chain), 1.78 (β, CH₂ in pDMAEMA and pHEMA main chain), 2.23 (η, 6H, CH₂-N-(CH₃)₂), 2.55 (ξ, 2H, CH₂-CH₂-N), 3.74 (ε, 2H, CH₂-CH₂-OH), 4.0 (γ, 2H of O-CH₂-CH₂ in pDMAEMA chain and δ, 2H of O-CH₂-CH₂ in pHEMA chain) ppm.

Second step. 2.0 g of DMAEMAₓ-HEMAᵧ were dissolved in 80 mL of THF under argon in each three-neck flask until complete dissolution of the copolymer. The flask was placed on an ice bath and 3.2 mL (for x:y ratio 3:1) or 6.8 mL (for x:y ratio 1:1) of triethylamine and 2.0 mL (x:y ratio 3:1) or 4.3 mL (x:y 1:1) of α-bromoisobutyryl bromide were sequentially added. The mixtures were then warmed to room temperature and left to react for additional two hours. The solutions were filtered through celite, then acidified with 50 mL of 1 M HCl. After removal of THF at the rotary evaporator, the solutions were neutralized at pH ~7 with 50 mL of 1 M sodium hydroxide, dialysed against distilled water and freeze-dried. Conversion (relative to alcohol groups): 97%. Yield (relative to a quantitative recovery of a 100% converted polymer): 55%.

1H NMR (D₂O): δ = 0.83-0.98 (α, CH₃ in pDMAEMA and pHEMA main chain), 1.86 (θ, 6H, C-(CH₃)₂-Br and β, CH₂ in pDMAEMA and pHEMA main chain), 2.81 (η, 6H,
Third step. Each copolymer was dissolved in 100 mL of water and the pH was brought to 9 by the addition of 1M sodium hydroxide. Subsequently 2 mL of methyl iodide were added under stirring, producing a white colloidal suspension which was kept reacting for 24 h and finally purified by dialysis against distilled water and freeze-dried. Conversion (relative to tertiary amine groups): 98%. Yield (relative to a quantitative recovery of a 100% converted polymer): 45%.

\[ \text{H NMR (D}_2\text{O): } \delta = 0.83-0.98 (\alpha, \text{ CH}_3 \text{ in pDMAEMA and pHEMA main chain}), 1.86 (\theta, 6\text{H, C-(CH}_3)_2-\text{Br and } \beta, \text{ CH}_2 \text{ in pDMAEMA and pHEMA main chain}), 2.81 (\eta_1, 9\text{H, CH}_2-\text{N-(CH}_3)_2), 2.97 (\gamma, 6\text{H, CH}_2-\text{N-(CH}_3)_2) 3.37 (\xi, 2\text{H, CH}_2-\text{CH}_2-\text{N}) 4.23-4.33 (\epsilon, 2\text{H, CH}_2-\text{CH}_2-\text{O-CO}, \gamma, 2\text{H of O-CH}_2-\text{CH}_2 \text{ in pDMAEMA chain and } \delta, 2\text{H of O-CH}_2-\text{CH}_2 \text{ in pHEMA chain})  4.75(2\text{H, H}_2\text{O traces}) \text{ ppm.} \]

ATR-IR (thin film): 3500-3100 (broad $\nu$ OH absorption, always present but variable intensity), 2960 and 2870 ($\nu$as CH$_3$ and CH$_2$), 1730 ($\nu$ CO), 1630 ($\delta$ H-O-H, always present but variable intensity), 1480 and 1370 ($\delta_{as}$ and $\delta_s$ CH$_3$ bending), 1275 and 1245, 1140-1160 ($\nu_{as}$ and $\nu_s$ C(=O)-O), 740 ($\nu$ C-Br) cm$^{-1}$.

### 2.3.4 Preparation of core-shell silica nanoparticles

#### 2.3.4.1 Preparation of silicagel nanoparticles

1 mL (4.4 mmol) of tetraethyl orthosilicate (TEOS) was mixed with 0.5 mL 1M hydrochloric acid; the pH dropped to 2.4, and the opaque suspension quickly turned into a homogeneous solution and was then left for two hours under stirring. Since the nucleation of silicic acid reaches a maximum speed at about neutral pH, 250 $\mu$L of PBS (10 mM, pH 7.4) were added to the hydrolyzed TEOS solution; different aliquots (20, 50, 100 and 200 $\mu$L) of the buffered solution were quickly mixed with 5 mL of 10 mM PBS containing 0, 0.001, 0.01 or 0.1 mg/mL horseradish peroxidase (HRP), to provide a final concentration of silicic acid of 0.6, 1.5, 3.0 or 6.0 $\text{SiO}_2$ mg/mL (expressing the
solid content as SiO$_2$ equivalents; they would correspond to 1.1, 2.1, 5.3 or 10.6 mg/mL of TEOS). Rapid (<1 hour) macroscopic gelation was observed for the two highest silicic acid concentrations; for all following experiments the silicagel nanoparticles were therefore produced at a concentration of 1.5 mg/mL.

### 2.3.4.2. Adsorption of macroinitiators on silica nano-substrates

10 mL of aqueous dispersions with different concentrations of LUDOX$^\textregistered$ (av. diameter 20 nm), silica nanoparticles (SiO$_2$-R-L1285, av. diameter 235 nm, Microparticles GmbH) or silicagel nanoparticles were added drop by drop at a flow rate = 1 mL/min by the help of a volumetric pump to 10 mL of aqueous solutions of macroinitiators (concentrations ranging between 0.025 and 1 mg/mL) under magnetic agitation (700 rpm). The resulting dispersions were then stirred for 15 minutes to allow adsorption to occur. The macroinitiator adsorption was monitored by dynamic light scattering and $\zeta$-potential analysis. Dispersions of coated nanoparticles were subsequently purified by gel filtration, eluting then through an aqueous sepharose column (Sephadex$^\textregistered$ G-50 for silica nanoparticles, Sephadex$^\textregistered$ G-25 for LUDOX and silicagel nanoparticles, flow rate = 1.5 mL/min, column diameter = 1.6 cm, column length = 20 cm) to remove the excess of macroinitiator. The efficiency of the separation was monitored on-line by following the absorbance of the eluted solution (230 nm) as a function of time, using a 50 $\mu$L flow-through cuvette (UV-Vis Lambda 12 spectrometer, Perkin Elmer). The concentrations of the final nanoparticle dispersions were measured as the solid content after freeze drying and resulted to be 0.09 % wt. (silica/DMAEMA$_{40}$-HEMA$_{40}$), 0.075 % wt. (silica/DMAEMA$_{24}$-HEMA$_{24}$), 0.10 % wt. (silicagel/DMAEMA$_{40}$-HEMA$_{40}$), and 0.09 % wt. (silicagel/DMAEMA$_{24}$-HEMA$_{24}$).

100 mL of a 0.015% wt. enzyme-containing silicagel nanoparticle dispersion (directly obtained from the above described preparation) were added to 100 mL of a 0.1% wt. macroinitiator solution, stirring gently for 15 minutes and then placing the dispersion into a dialysis tube (MWCO=3,500 Da) to remove the excess of buffer salts. The resulting 200 mL were concentrated by ultrafiltration (MWCO 100 KDa) down to 10 mL (concentration = 0.15% wt, always expressed in weight of SiO$_2$, which is likely to
be a gross underestimation of the solid content of the dispersion) and purified by Sephadex G-25 and two different fractions were collected monitoring the separation by UV spectroscopy: the first fraction (16 mL) was freeze dried in order to determine the actual concentration of silicagel in the fraction, which resulted to be 0.12 % wt. for silicagel/HRP/DMAEMA$_{40}$-HEMA$_{40}$ and 0.11 % wt. for silicagel/HRP/DMAEMA$_{24}$-HEMA$_{24}$.

ATR-IR (thin film): 3600-300 (broad ν OH absorption, variable intensity), 2960 (ν$_{as}$ CH$_3$ and CH$_2$), 2820 and 2770 (ν NH (units quaternarized through protonation rather than alkylation) + other stretching vibrations of ammonium ions), 1725 (ν C=O), 1630 (δ$_{as}$ and δ$_s$ H$_2$O bending, always present but variable intensity), 1450 and 1370 (δ$_{as}$ and δ$_s$ CH$_3$ bending), 1275 and 1245, 1130-1160 (ν$_{as}$ and ν$_s$ C(=O)-O), 1080-1100 (ν Si-O-Si) cm$^{-1}$.

2.3.5 Polymerization experiments (glycerol monomethacrylate GMMA)

2.3.5.1. ATRP in solution using EBiB as a model initiator

In a typical experiment, the polymerization reaction was run at 20°C, in a DMF:H$_2$O 50:50 (v/v) solvent mixture, using [GMMA] = 2 M, [GMMA]:[EBiB]:[CuCl] = 200:1:1, [CuCl]:[CuCl$_2$] = 1:1.2, 1:0.8, 1:0.4 and 1:0, and [bpy]/([CuCl] + [CuCl$_2$]) = 2.5. 1.6 g of GMMA (10 mmol), 7.4 mL of 7EBiB (0.05 mmol), 2.25 mL of water and 2.5 mL of DMF were introduced into a Schlenck tube and the mixture was degassed by purging it with argon for 10 minutes. A catalyst water stock solution was prepared adding 1 mL of degassed water to 20 mg (0.2 mmol) of CuCl and different quantities of CuCl$_2$ and bpy (see Table 2.1) under argon. After withdrawing an initial sample to measure the monomer concentration at time $t_0$, 0.250 mL of the freshly prepared CuCl-CuCl$_2$-bpy stock solution were added to the GMMA solution. The polymerization was sampled at suitable time periods throughout the reaction. Samples for the kinetic study (10 μL) were diluted in 8 mL of H$_2$O:CH$_3$CN (90:10 v/v) and directly injected in the HPLC column.
Similar conditions were adopted for the polymerization of GMMA using \([\text{CuCl}]:[\text{bpy}]\) 1:2.5 but varying the DMF:HzO ratio from 25:75 (v/v), 50:50 (v/v) to 75:25 (v/v); same procedure was exploited to monitor the monomer consumption throughout the reaction.

Experimental conditions are summarized in table 2-1.

<table>
<thead>
<tr>
<th>GMA (g/mmol/eq)</th>
<th>EBiB (mL/mmol/eq)</th>
<th>CuCl (mg/mmol/eq)</th>
<th>CuCl2 (mg/mmol/eq)</th>
<th>Byp (mg/mmol/eq)</th>
<th>DMF (mL)</th>
<th>HzO (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6/10/200</td>
<td>7.4/0.05/1</td>
<td>5/0.05/1</td>
<td>-</td>
<td>20/0.125/2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>1.6/10/200</td>
<td>7.4/0.05/1</td>
<td>5/0.05/1</td>
<td>2.5/0.02/0.4</td>
<td>27.3/0.175/2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>1.6/10/200</td>
<td>7.4/0.05/1</td>
<td>5/0.05/1</td>
<td>5.5/0.04/0.8</td>
<td>35/0.225/2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>1.6/10/200</td>
<td>7.4/0.05/1</td>
<td>5/0.05/1</td>
<td>8/0.06/1.2</td>
<td>43/0.275/2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

2.3.5.2. ATRP in solution on macroinitiators

Typical polymerization conditions: DMF:HzO 50:50 (v/v) at 20 \(\degree\)C, [GMMA] 2 M, [GMMA]:[Initiator groups]:[CuCl] = 200:1:1, [CuCl]:[CuCl2] = 1:0.8 and \([\text{bpy}]/([\text{CuCl}]+[\text{CuCl2}]) = 2.5\).

Each of the macroinitiators was weighed [25 mg, 0.05 mmol of initiator groups] considering 10% of water content and introduced in a Schlenk tube; GMMA (1.6 g, 10 mmol), 2.25 mL of water and 2.5 mL of DMF were added and degassed purging with argon. A CuCl-CuCl2-bpy water stock solution was prepared adding 1.5 mL of degassed water to 30 mg (0.30 mmol) of CuCl, 33 mg CuCl2 (0.24 mmol) and 210 mg (1.35 mmol) of bpy under argon. After withdrawing the initial sample at time t=0 sec, 0.250 mL of the catalyst stock solution were added to the GMMA solution. The polymerization was sampled at suitable time periods throughout the reaction. Samples
for the kinetic study (10 μL) were diluted in 8 ml of H$_2$O:CH$_3$CN (90:10 v/v) and directly injected in the HPLC column.

### 2.3.5.3. Surface-initiated ATRP on silica nanoparticles

Typical conditions for a polymerization experiment: DMF : H$_2$O 50:50 (v/v) at 20 °C, [GMMA] = 2 M, [GMMA]:[initiator on SiO$_2$ nanoparticles]:[CuCl] = 125 or 165:1:1, [CuCl]:[CuCl$_2$] = 1:0.8 and [bpy]/([CuCl] + [CuCl$_2$]) = 2.5. 0.32 g GMMA (2 mmol), 0.5 mL of a nanoparticle water dispersion (2.97 and 2.47% wt., respectively for nanoparticles coated with pDMAEMA$_{40}$-pHEMA$_{40}$ and with pDMAEMA$_{24}$-pHEMA$_{24}$, corresponding to 0.016 and 0.012 mmol of initiator under the assumptions that the organic content is totally attributable to the macroinitiator and the loss of halogens during adsorption does not significantly affect the mass of the polymer) and 0.3 mL of DMF were introduced in a Schlenk tube and degassed purging with argon. A CuCl-CuCl$_2$-bpy stock solution was prepared adding 2 mL of degassed DMF to 10 mg (0.1 mmol) of CuCl, 11 mg (0.08 mmol) of CuCl$_2$ and 70 mg (0.45 mmol) of bpy under argon. Before introducing the catalyst in the reaction tube, 0.1 mL of solution were sampled and diluted with 0.9 mL of H$_2$O for the DLS analysis; similarly 10 μL were withdrawn and diluted in 8 ml of H$_2$O:CH$_3$CN (90:10 v/v) for the HPLC analysis. Finally 0.2 mL of the catalyst solution were added to the tube and the reaction was monitored by sampling 0.1 mL (diluted with 0.9 mL of H$_2$O) and 10 μL (diluted in 8 mL of H$_2$O:CH$_3$CN) aliquots at suitable time intervals to evaluate nanoparticle size and ζ-potential and monomer conversion; the polymerization was then stopped by bubbling air and the mixture was purified by extensive dialysis against distilled water.

### 2.3.5.4. Surface-initiated ATRP on silicagel nanoparticles

Typical conditions for a polymerization experiment: DMF:H$_2$O 50:50 (v/v) at 20 °C, [GMMA] = 1 M, [GMMA]:[initiator on silicagel nanoparticles]:[CuCl] = 100:1:1, [CuCl]:[CuCl$_2$] = 1:0.8 and [bpy]/([CuCl] + [CuCl$_2$]) = 2.5. 0.32 g GMMA (2 mmol), 1 mL of a nanoparticle dispersion (2.24 and 2.13% wt., respectively for nanoparticles coated with DMAEMA$_{40}$-pHEMA$_{40}$ and with DMAEMA$_{24}$-pHEMA$_{24}$, corresponding to 0.02 mmol of initiator under the assumptions that the organic content is totally attributable to
the macroinitiator and the loss of halogens during adsorption does not significantly affect the mass of the polymer) and 0.8 mL of DMF were introduced in a Schlenck tube and degassed purging with argon. A CuCl-CuCl₂-bpy stock solution was prepared adding 2 mL of degassed DMF to 10 mg (0.1 mmol) of CuCl, 11 mg (0.08 mmol) of CuCl₂ and 70 mg (0.45 mmol) of bpy under argon. Samples were collected as described above and the polymerization was finally stopped by bubbling it with air, and the mixture purified by extensive dialysis against distilled water.

### 2.4 Results and Discussions

#### 2.4.1 Synthesis of macroinitiators

Adopting a strategy of polyelectrolyte surface complexation, we have synthesized a series of cationic macroinitiators to decorate the surface of negatively charged silica particles, following the approach first reported by Armes et al. [83]. Two factors were taken into account in designing the copolymer: (i) the cationic macroinitiator should be water-soluble to allow electrostatic adsorption in aqueous solutions and its molecular weight should be relatively low to avoid excessive bridging flocculation of the anionic silica nanoparticles. Accordingly, we have used quaternized DMAEMA as the cationic monomer, in stoichiometric equivalence or in excess compared to the other monomer, i.e. HEMA esterified with an ATRP initiator. Such polymers were produced (Figure 2-2) by copolymerizing HEMA and DMAEMA via ATRP in methanol at 20 °C with a Cu(I)Br/bpy catalyst.
Figure 2-2. Reaction scheme for the three-step synthesis of the cationic macroinitiator used for the surface ATRP of glycerol monomethacrylate onto Silica NPs.

The copolymerization proceeded to very high conversion and the DMAEMA/HEMA molar ratio of the isolated precursor copolymer was found to be very close to the target copolymer composition by $^1$H NMR spectroscopy (see appendix A). The hydroxyl groups of HEMA residues were then esterified using an excess of 2-bromoisobutyryl bromide and finally the DMAEMA residues were quantitatively quaternarized by the use of methyl iodide. The characterization data are summarized in Table 2-1. It is worth pointing out that a) the higher-than-expected molecular weight values are possibly due to the use of poly(methyl methacrylate) standards: their smaller hydrodynamic volume may cause an overestimation in the molecular weight determination; additionally, some aggregation in the GPC solvent (DMF) cannot be completely ruled out; b) although the degrees of esterification and quaternization were substantially quantitative, the overall yield for the macroinitiators was only approximately 25%, due to losses incurred during its purification.
### Table 2.2. Summary of the characterization data for the cationic macroinitiators synthesized in this work.

<table>
<thead>
<tr>
<th>Macroinitiator</th>
<th>Theoretical DP and (g/mol)</th>
<th>Monomer molar ratio$^a$</th>
<th>$\overline{M}_n$ b (g/mol)</th>
<th>$\overline{M}_w$ b (g/mol)</th>
<th>$\overline{M}_w / \overline{M}_n$</th>
<th>D.E. (%) c</th>
<th>D.Q. (%) c</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMAEMA$<em>{12}$-HEMA$</em>{12}$</td>
<td>24 / 3450</td>
<td>1:0.98</td>
<td>6280</td>
<td>7950</td>
<td>1.27</td>
<td>96.2</td>
<td>Quant.</td>
</tr>
<tr>
<td>DMAEMA$_{18}$-HEMA$_6$</td>
<td>24 / 3600</td>
<td>3:1.15</td>
<td>4650</td>
<td>5720</td>
<td>1.23</td>
<td>95.8</td>
<td>Quant.</td>
</tr>
<tr>
<td>DMAEMA$<em>{24}$-HEMA$</em>{24}$</td>
<td>48 / 6900</td>
<td>1:0.96</td>
<td>12470</td>
<td>15670</td>
<td>1.25</td>
<td>97.5</td>
<td>Quant.</td>
</tr>
<tr>
<td>DMAEMA$<em>{36}$-HEMA$</em>{12}$</td>
<td>48 / 7200</td>
<td>3:0.93</td>
<td>9970</td>
<td>11960</td>
<td>1.20</td>
<td>98.1</td>
<td>Quant.</td>
</tr>
<tr>
<td>DMAEMA$<em>{40}$-HEMA$</em>{40}$</td>
<td>80 / 11500</td>
<td>1:1.1</td>
<td>16000</td>
<td>20950</td>
<td>1.32</td>
<td>97.3</td>
<td>Quant.</td>
</tr>
<tr>
<td>DMAEMA$<em>{60}$-HEMA$</em>{30}$</td>
<td>80 / 12040</td>
<td>3:0.95</td>
<td>11100</td>
<td>13650</td>
<td>1.23</td>
<td>96.4</td>
<td>Quant.</td>
</tr>
</tbody>
</table>

$^a$ DMAEMA/HEMA molar ratio in the polymer calculated from $^1$H-NMR spectra.

$^b$ From GPC analysis.

$^c$ Degree of esterification (D.E.) and of quaternization (D.Q.) calculated from $^1$H-NMR spectra respectively on non-quaternized polymers dissolved in deuterated chloroform and quaternized polymers dissolved in deuterated water.

### 2.4.2 Optimization of the preparative and purification processes for coated nanoparticles

We aimed to prepare enzyme-containing silicagel nanoparticles through an aqueous sol-gel process, then decorating them through surface-initiated ATRP. The silicagel nanoparticles were prepared through a simple aqueous procedure of acid hydrolysis and condensation at neutral pH (a sol-gel process) of tetraethyl orthosilicate (TEOS). This process leads to macroscopic gelation at high TEOS concentration; low concentrations can drive this process to the formation of nanoparticles with a general composition SiO$_2$-$\alpha$(OH)$_{2\alpha}$, i.e. an imperfect and porous silica network, which we refer to as silicagel. The process can be performed also in the presence of enzymes, leading to the nanoparticles with slightly lower dimensions (Z-average size decreasing from 99 nm to 62, Figure 2-3) and increased $\zeta$-potential (from -45 to -22 mV, for a comparison see...
Figure 2-4). The degree of incorporation of the enzyme was difficult to assess, but it was assumed to be substantially quantitative: dialysis of the nanoparticles (MWCO = 50,000 g/mol, HRP MW is approx. 44,000 g/mol) showed negligible enzymatic activity in the dialysate. This would correspond to a theoretical HRP content in the nanoparticle of about 6% wt. considering the nanoparticles as pure SiO₂; since, however, the nanoparticles have a silicagel structure, comprising silanol groups and tightly bound water, it is reasonable to foresee a lower enzyme content. TGA analysis (data not showed) suggested the presence of about 1-2% wt. of organic material, although the measure were extremely noisy because the weight loss was overwhelmingly dominated by the water loss of silicagel.

Figure 2-3. Size distributions for silicagel nanoparticles (concentration: 1.5 mg SiO₂ / mL) prepared in the presence of increasing concentrations of HRP.

In comparison to the more popular inverse emulsion route to silica nanoparticles [84, 85], which was used in the past also by our group [1], and to the TEOS hydrolysis in homogeneous basic hydroalcoholic solution [86, 87], this all-aqueous method has some key advantages: absence of possibly denaturing organic solvents and emulsifiers, and overall simplicity. As a drawback, these nanoparticles feature limited stability and tend
to irreversibly agglomerate in the time frame of a few hours at physiological pH, unless a self-repellent shell, e.g. a polycation layer, is quickly provided.

Depending on the conditions of the process, the adsorption of cationic polymers can lead to the flocculation of the negatively charged silica colloidal substrate, with the polycations bridging two or more particles.

We have here investigated the influence of macroinitiator composition (1:1 and 3:1 DMAEMA/HEMA molar ratio) and molecular weight (theoretical DP = 24, 48 and 80), and of the concentration of both macroinitiators and nanoparticles, with the aim to possibly find robust conditions leading to the nanoparticle decoration. We have also aimed at identifying a rapid purification procedure that could quantitatively exclude non-adsorbed macroinitiators.

We have used two model substrates for a preliminary screening of the preparative and purification procedures. The two model systems, respectively with larger and smaller nanoparticles compared to the target silicagel ones, are commercially available: LUDOX® TMA colloidal silica (Z-average size: 33 nm, ζ-potential: -20 mV in deionized water), and near-monodisperse colloidal silica (SiO₂-R-L1285, Z-average size: 234 nm, ζ-potential: -54 mV in deionized water). Representative distributions for both size and ζ-potential of these three silica model systems are provided in Figure 2-4.

The adsorption of macroinitiators on silica nanoparticles (Figure 2-5) caused a small increase in particle size, from 235 to 250-270 nm. This increase was slightly larger at higher polymer concentrations, an effect possibly due to a higher density of the polymer brushes and therefore their more extended character of the polymer brushes, rather than to particle agglomeration. The structure of the macroinitiator (ratio between charged and uncharged monomer, molecular weight) did not appear to influence the size of the coated particles, while a slightly higher ζ-potential (~ +80 mV vs. ~ +70 mV) was noticed upon adsorption of the macroinitiators with a higher content of ammonium ions.
Figure 2-4. Size (left) and ζ-potential (right) distributions for the silica-based colloidal substrates employed in this study. The HP silicagel nanoparticles were prepared from 0.1 mg/mL HRP and a silicagel nominal concentration of 1.5 mg SiO$_2$/mL.

A similar, negligible influence of the macroinitiator structure could be seen on the Ludox nanoparticles (Figure 2-6), with a few differences probably arising from their smaller size. In addition to obtaining a slightly lower ζ-potential, +60-65 mV and +50-60 mV respectively for the adsorption of the polymers with a higher or lower content of ammonium ions, the process of coating caused in several cases significant agglomeration of Ludox nanoparticles; this was particularly evident for the lowest Ludox concentration (0.01 % wt.) and at high polymer concentrations (>0.1 % wt.), where bimodal distributions comprising particles sized in the hundreds of nanometers were often recorded. Last, more evidently for the silica nanoparticles the ζ-potential seemed to reach a plateau at polymer concentrations ≥0.1% wt.
Figure 2.5. Z-average size (above) and average ζ-potential (below) of macroinitiator-coated silica nanoparticles as a function of the coating conditions. Concentrations of macroinitiator and silica nanoparticles are reported in table 2.3.
Figure 2-6. Z-average size (above) and average ζ-potential (below) of macroinitiator-coated Ludox nanoparticles as a function of the coating conditions. A relevant number of samples, mostly related to adsorption experiments with the lowest polymer concentration, black triangles, are not reported in the upper part of the figure, due to the presence of substantial aggregation (bimodal size distributions). Concentrations of macroinitiator and silica nanoparticles are reported in table 2-4.
As a result of this phase of optimization, we reached the following conclusions:

A) The composition and molecular weight of the macroinitiator did not have any noticeable influence on the size and charge of the coated nanoparticles. In order therefore to maximize the amount of initiators on the nanoparticle surface, we have selected the 1:1 composition for any further experiment. We have decided to employ the polymers with the two highest molecular weights (DMAEMA\textsubscript{40}-HEMA\textsubscript{40} and DMAEMA\textsubscript{24}-HEMA\textsubscript{24}), in order to ensure the stability of the adsorbed layers.

B) The effect of the polymer and nanoparticle concentrations on the nanoparticles stabilization was moderate. The choice of experimental conditions depended therefore on practical considerations: in order to facilitate the purification from free macroinitiators, we have adopted the lowest polymer concentration that allowed to obtain plateau values of $\zeta$-potential (0.1 % wt.). For what attains to nanoparticles, for silica and Ludox we have adopted the highest concentration (0.1 %wt.), in order to maximize the amount of adsorbed polymer. On the other hand, silicagel nanoparticles could be produced only at relatively high dilution, in order to avoid macroscopic gelation during their preparation, while their limited stability did not allow any concentration procedure, e.g. via ultrafiltration. These nanoparticles were used at a concentration of 0.015 % wt. of SiO\textsubscript{2}; however, since the stoichiometry of silicagel should include silanol groups too and its weight should consider also large amounts of entrapped water, one must realize that their real concentration is considerably higher and possibly closer to that of the other nanoparticular systems. At this concentration, the coating of silicagel nanoparticles showed a qualitative similarity to that of silica and Ludox ones (Figure 2-7).

The purification of all nanoparticles prepared in this study was performed through gel filtration on sepharose columns. Compared to other techniques such as dialysis or ultrafiltration, gel filtration is significantly advantageous in terms of rapidity and ease of evaluation of the product purity.
Indeed, the efficiency of the process could be easily monitored on-line with the help of UV-Vis spectroscopy (Figure 2-8), or off-line, by recording thermogravimetric scans (Figure 2-8) or FT-IR spectra (Figure 2-9) on isolated fractions; also the enzymatic activity of the eluted fractions can be used to determine the efficiency of the purification of enzyme-containing nanoparticles (Figure 2-9). However, even using Sepharose with the smallest pore size (Sephadex® G-25), the purification of Ludox nanoparticles was unsuccessful, possibly due to their small difference in size with the macroinitiators. For this reason, Ludox nanoparticles were at this point abandoned and the study focused on silica and (enzyme-containing) silicagel nanoparticles, separated from macroinitiators.
respectively with Sephadex® G-25 (Fractionation range (MW) = 1,000-5,000 for globular proteins, 100-5,000 for dextrans) and G-50 (Fractionation range (MW) = 1,500-30,000 for globular proteins, 500-10,000 for dextrans).

Figure 2-8. TGA curves and elution profiles (in the inserts) for as-prepared and purified silica nanoparticles (above) and silicagel nanoparticles (below). Both systems were coated using a 0.1 % wt. solution of DMAEMA₄₀-HEMA₄₀. During the TGA runs the samples were kept for one hour at 120°C for the complete evaporation of water.
Figure 2-9. Top. IR spectra of DMAEMA\textsubscript{40}-HEMA\textsubscript{40} (macroinitiator) and silica nanoparticles before and after purification (first peak) through gel filtration. In comparison to the intensity of the absorption for the Si-O stretching vibration (roughly 1100 cm\textsuperscript{-1}), the carbonyl stretching absorption, related to the amount of macroinitiator, is drastically reduced after purification. Middle. A similar pattern can be seen in the IR spectra of silicagel nanoparticles, with a decreased amount of DMAEMA\textsubscript{40}-HEMA\textsubscript{40} in the first fraction and a larger amount in the second fraction. Bottom. Enzyme kinetics of Horse Radish Peroxidase for as-prepared (solid line) silicagel nanoparticles and the two fractions obtained after gel filtration. Conditions: T = 20° C, pH = 6.0, A = 420 nm, light path = 1cm. The enzyme activity (U/ HRP mL) was calculated from the initial linear slope considering that 1 Unit/mL of enzyme converts 1.0 mg of pyrogallol in purpurogallin in 20 seconds.
It is worth pointing out that the activity of HRP in the purified silicagel nanoparticles appeared comparable to that of the pure enzyme, i.e. in the range of hundreds of units per mg of enzyme. Also the morphology of the nanoparticles was not appreciable altered after purification; on mica surfaces the coated silica nanoparticles revealed a smoother surface and a somehow flattened shape, while the silicagel ones showed a more rough and “rocky” form (Figure 2-10).

![Figure 2-10. Tapping mode AFM pictures of coated (DMAEMA$_{40}$-HEMA$_{40}$) silica and silicagel nanoparticles before and after purification through gel filtration (Sepharose G-50 and G-25, respectively). The presence of other components, e.g. free macroiniators, is particularly evident in the bottom left picture.]

Gel filtration did not appreciably influence the size or $\zeta$-potential of the nanoparticles, but considerably lowered the organic content of the dispersions, as it can be seen in the last column of Table 2-3. It is worth pointing out that, although the gel filtration elution...
provided monomodal traces, suggesting a complete removal of free macroinitiators, the still relatively high organic content of the dispersions would suggest the presence of either a thick rather than monomolecular layer on the silica surface, or of some remaining free macroinitiator.

Table 2-3. Summary of the characteristics of the optimized coated nanoparticles

<table>
<thead>
<tr>
<th>Macroinitiator</th>
<th>Z-average size (before/after) a (nm)</th>
<th>Av. ζ-potential (before/after) a (mV)</th>
<th>Nanoparticle concentr. (% wt.)</th>
<th>Polymer concentr. (% wt.)</th>
<th>Org. fract. (before/after) (% wt.) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ludox</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMAEMA_{24}−</td>
<td>35/68</td>
<td>-20/+63</td>
<td>0.1</td>
<td>0.1</td>
<td>=</td>
</tr>
<tr>
<td>HEMA_{24}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMAEMA_{40}−</td>
<td>35/76</td>
<td>-20/+51</td>
<td>0.1</td>
<td>0.1</td>
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</tr>
<tr>
<td>HEMA_{40}</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silica</td>
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<td></td>
</tr>
<tr>
<td>DMAEMA_{24}−</td>
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<td>0.1</td>
<td>61/27</td>
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<td>HEMA_{24}</td>
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<tr>
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<td>234/259</td>
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<td>0.1</td>
<td>56/32</td>
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<td>HEMA_{40}</td>
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<td>Silicagel</td>
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<td>DMAEMA_{24}−</td>
<td>99/136</td>
<td>-45/7</td>
<td>0.015 c</td>
<td>0.1</td>
<td>58/30</td>
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<td>0.1</td>
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<td>0.1</td>
<td>57/29</td>
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<tr>
<td>HEMA_{24}</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>DMAEMA_{40}−</td>
<td>65/88</td>
<td>-25/15</td>
<td>0.015 c</td>
<td>0.1</td>
<td>58/24</td>
</tr>
<tr>
<td>HEMA_{40}</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

a Data refer to the size and ζ-potential before and after the macroinitiator adsorption.

b Organic fraction of Silica nanoparticles determined by thermogravimetric analysis, before and after purification, as the weight loss by 800°C under nitrogen atmosphere at a scan rate of 10°C/min. Data refer to the organic fraction before and after the purification via gel filtration.

c Nanoparticle concentration after macroinitiator adsorption.
2.4.3 ATRP experiments in solution

Choice of the initiator. One of the best initiators for methacrylates, \( p \)-toluenesulfonyl chloride, would react with the hydroxy functionality of 2-hydroxyethyl methacrylate or glycerol monomethacrylate \[^{[88]}\]. 2-bromopropionates, a widely used class of ATRP initiators, are generally not suitable for methacrylate polymerizations, because of their slow initiation \[^{[89]}\]. 2-bromoisobutyrates, are possibly the most popular ATRP initiators, and are also the groups with the closest similarity to the structure of the propagating methacrylate chain end \[^{[5]}\]; although their stability depends on the nature of the initiation they are stable under aqueous reaction conditions and soluble in the reaction medium (water/DMF mixture). We have therefore selected ethyl \( \alpha \)-bromoisobutyrate (EBiB) as initiator for all polymerization experiments and it was always used in equimolar amounts to \( \text{Cu(I)} \), which was always in the form of \( \text{CuCl} \), due to its well established efficiency in ATRP \[^{[90],[91],[92]}\].

Choice of the solvent. Poly(glycerol monomethacrylate) is soluble only in water and other highly polar solvents or solvent mixtures. Water / \( N,N \)-dimethylformamide (DMF) mixtures are particularly interesting, since they combine protic character, the possibility to tune polarity through a wide range of dielectric constants (between 38 (DMF) and 81 (water)), and also the ability to solubilize rather hydrophobic polymers, thereby also allowing in perspective the preparation of amphiphilic block copolymers of GMMA. The presence of tertiary amides (DMF) during ATRP has been questioned since nitrogen atom may compete with the ligand in the catalyst complexation reducing its activity; indeed the complexation of the copper catalysts by DMF residues has been shown to unbalance the activation/deactivation equilibrium increasing the concentration of radical species at the expenses of C-Br groups, causing the loss of control of the polymerization \[^{[93]}\]. However, this effect appears to be present only for tertiary amides present along the polymer chains; on the contrary DMF \[^{[94]-[97]}\] or water/DMF mixtures \[^{[98]-[101]}\] have widely been used to carry out well-controlled ATRP of methacrylates and also of the more problematic acrylamides (\textit{vide supra}), the latters generally through the use of polydentate amine ligands and the addition of \( \text{CuCl}_2 \) \[^{[102]}\]. It should be pointed out that the loss of the chloride ligand from the deactivator in the
presence of water or other possibly coordinating compounds, such as DMF, can be suppressed by the addition of chlorides allowing the regeneration of the dissociated Cu(II)Cl₂ species, or simply adding a sufficiently large amount of Cu(II)Cl₂, thereby increasing the deactivation rate. It is noteworthy that the above considerations apply not only to ATRP but also to the closely related single-electron transfer living radical polymerization (SET-LRP) [103].

We have initially carried out experiments to find the water/DMF ratio that would allow a good control over GMMA polymerization in solution, while maximizing the polymerization rate. These preliminary experiments (Figure 2-11, left) were conducted in the absence of CuCl₂, whose presence and concentration was optimized in a second stage. All the solvent mixtures provided very high conversions, up to 90-95%; however, ln([M]₀/[M]) showed a pronounced non-linearity with time, indicating a significant incidence of termination events possibly due to a high radical concentration.


The: 25:75 (v/v) DMF:water mixture resulted in a more evident loss of linearity, although a fairly high conversion of 85% was achieved in 100 minutes; large concentrations of water are known to increase the propagation rate K_p (stronger hydrogen bonds formed between the polar monomer and water), while the complexation of copper with water (aquation) depletes the deactivator concentration throughout the polymerization.
The loss of linearity was much less pronounced for 50% and 75% DMF mixtures, with a clear deceleration of the reaction for the highest DMF content. We have therefore selected the 50% mixture for further experiments, as a compromise between polymerization rate and controlled character.

**Choice of the catalytic system.** Employing a classical 2,2’-bipyridine (bpy) ligand, we have thus optimized the polymerization conditions in terms of the amounts of CuCl$_2$ suitable to improve the control over the polymerization. The experiments were performed in a water:DMF 50:50 (v/v) mixed solvent at room temperature, with a constant 2.5 molar ratio between bpy and the total amount of copper species [48].

All the polymerizations (Figure 2-10, right) presented very high conversions, up to 95-99%; the use of [CuCl$_2$]$_0$/[CuCl]$_0$ ratios of 0.8 and 1.2 showed a reasonable linear dependence of ln([M]$_0$/[M]) with time, up to a conversion of 88% (314 min) and 72% (544 min) respectively, suggesting a constant concentration of propagating species and therefore negligible termination; on the contrary polymerizations carried out without CuCl$_2$ or with a [CuCl$_2$]$_0$/[CuCl]$_0$ ratio of 0.4 resulted in a clear loss of linearity at high conversions, which were reached in only 30-40 minutes.

This is not unexpected: at high Cu(II) / Cu(I) ratios, the equilibrium is shifted towards the dormant species, reducing the radical concentration, and thus slowing the polymerization down and limiting the incidence of termination events. Our results are in agreement with what reported by Tsarevsky et al. [62]: to achieve a controlled polymerization in protic media, an excess halide salts is required to “regenerate” the dissociated deactivator.

**Graft polymerization on macroinitiators.** In order to highlight possible specific effects arising from the polycationic nature of the initiators and/or from the high local density of initiators, we have conducted the ATRP of GMMA on DMAEMA$_{24}$-HEMA$_{24}$ and DMAEMA$_{40}$-HEMA$_{40}$, in 50:50 (v/v) DMF:water as a solvent, with a [CuCl$_2$]$_0$ / [CuCl]$_0$ ratio of 0.8 and with the same monomer/initiator groups used with (Figure 2-12). In comparison to the initiation by EB/B, both macroinitiators produced a significant, although not dramatic, decrease in the monomer consumption rate, which however did not affect the linearity of ln([M]$_0$/[M]) with time. Surprisingly, the longer macroninitiator showed a slightly higher activity than the shorter one, thus showing that electronic factors or the local dielectric constant overwhelm the possibly steric
hindrance of a longer polymer chain. In both cases the increase in the solution viscosity was more pronounced than in the case of initiation through EBiB and the mixtures gelled after about four hours of polymerization; the gelation, however, could be reversed by addition of water or other polar solvents, demonstrating that the phenomenon was caused by the entanglements between branched macromolecular structures rather than by covalent cross-links.

![Figure 2-12](image)

**Figure 2-12.** Kinetic plots for the ATRP of GMMA on different initiator systems. Conditions: water:DMF 50:50 (v/v), $T = 20 \, ^\circ\text{C}$, $[\text{GMMA}]_0:[\text{initiator groups}]_0:[\text{CuCl}]_0:[\text{CuCl}_2]_0:[\text{bpy}]_0 = 100:1:1:0.8:4$, $[\text{GMMA}]_0 = 1 \, \text{M}$. Error bars calculated on three repeats. ATRP of GMMA initiated by the two cationic macroinitiators and by EBiB as a comparison. It is apparent that the polymerization maintains its living character slowing down in the order EBiB > DMAEMA$_{40}$-HEMA$_{40}$ ≥ DMAEMA$_{24}$-HEMA$_{24}$.

### 2.4.4 ATRP on nanoparticles

The kinetics of surface-initiated polymerization on silica nanoparticles and that on silicagel ones showed significant differences. Although similarly rapid until about 40% monomer consumption, at higher conversions the polymerization on silica nanoparticles
showed then a sharp decrease in rate, while that on silicagel nanoparticles continued with a rapid kinetics until the solution gelled with only a slight curvature of $\ln([M]/[M])$ with time (Figure 2-13). No difference between the two macroinitiators was observed for silicagel nanoparticles, nor in the first part of the kinetics for silica nanoparticles.

Figure 2-13. Kinetic plots for the ATRP of GMMA on different nanoparticulate systems. Conditions: water:DMF 50:50 (v/v), $T = 20$ °C, $[\text{GMMA}]_0:[\text{initiator groups}]_0:[\text{CuCl}]_0:[\text{CuCl}_2]_0:[\text{bpy}]_0 = 100:1:1:0.8:4$, $[\text{GMMA}]_0 = 1$ M. Error bars calculated on three repeats. ATRP of GMMA on the different; the early time points are shown with magnified scales in the insert. Silicagel nanoparticles showed an extremely fast polymerization kinetics, much more rapid than any of the cases recorded in solution; the presence of encapsulated HRP did not appear to influence the kinetics. The polymerization on silica nanoparticles had a similar start, but quickly reached a second phase of much slower monomer consumption.

Silicagel nanoparticles showed a significant although not dramatic increase in dimension and a decrease in $\zeta$-potential, respectively to 150 - 200 nm and about 0 mV, irrespectively of the nature of macroinitiator and of the presence of HRP (Figure 2-14): such increase in particle diameter and decrease of zeta potential suggest a good effective steric stabilization provided by the pGMMA external layer.
Figure 2-14. Size (left) and \( \zeta \)-potential (right) distributions for the silicagel nanoparticles before (solid line) and after (dashed line) polymerization of GMMA. The polymerization time was kept to 25 minutes to avoid gelation and allow an easy sampling of the dispersions.

Also silica nanoparticles showed the expected progressive reduction in \( \zeta \)-potential with increasing polymerization time, with negligible differences between the two macroinitiators (Figure 2-15, bottom); the \( \zeta \)-potential approached neutrality roughly at the same time (about 30’) when the conversion of both systems showed a clear deviation from linearity. Size too appeared to grow significantly (up to about a Z average of 300 nm) only until a similar time point (Figure 2-15, top); interestingly, at longer times the DMAEMA\(_{40}\)-HEMA\(_{40}\)-coated nanoparticles showed an increasingly bimodal distribution. We interpret the lower size peak as due to the polymerization in the solution phase initiated by some free or desorbed initiators, possibly not totally removed during gel filtration. The size distribution of DMAEMA\(_{24}\)-HEMA\(_{24}\)-coated nanoparticles did not appear to show a bimodal character, but this does not exclude the formation of soluble materials, which may not be recorded either because of the smaller concentrations or, more likely, because of the lower difference in refractive index: a smaller macroinitiator/GMMA ratio would significantly reduce the scattered intensity per volume unit.
Figure 2-15. Size (top) and Zeta potential distributions (bottom) for silica nanoparticles coated with two macromonomers as a function of time during the ATRP of GMMA. A bimodal size distribution becomes evident at long polymerization times for the nanoparticles covered with pDMAEMA40-pHEMA40; however, the corresponding monomer conversion, i.e. the amount of polymer produced, is relatively small (see Figure 2-11, right).
It seems therefore reasonable to suppose that polymerizations initiated from silica nanoparticles had proceeded similarly to those on silicagel ones until a substantial surface coverage was obtained. The average particle diameter increased progressively over the polymerization until saturation is achieved (figure 2-16, top); zeta potential average over the time show a similar saturation behaviour (figure 2-16, bottom) suggesting a highly effective steric stabilization provided by the pGMMA external layer.

At this point, while some monomer would be consumed through polymerization on free macroinitiators, the surface-initiated polymerization would then appear to substantially stop, although it is unclear whether this may be caused by steric reasons or by sequestration of metal centres.

Figure 2-16. Diameter (top) and Zeta potential average values (bottom) for silica nanoparticles coated with two macroiniators as a function of time during the ATRP of GMMA.
According to DLS and Zeta potential, the poly(GMMA) film should have a thickness in the order of a few tens of nanometers, i.e. thick enough to shield the charge of the underlying silica surface, which was qualitatively confirmed by AFM (Figure 2-17).

However it is noteworthy to point out that no calculation of the graft density was feasible from the combination of DLS and AFM data because of the porosity of the silica nanoparticles, thus no evaluation of the surface area was achievable.

![Figure 2-17. Tapping mode AFM images of silica nanoparticles during GMMA polymerization. During polymerization, the nanoparticles appear modified by the presence of external softer material. Although images at intermediate times (below 100 minutes) show a more “patchy” appearance (see the image in the middle), the amount of this material does not appear to have a sound increase with time.](image)

The very different behaviour shown by silicagel nanoparticles is possibly due to a more “open” structure. Silicagel, i.e. the material obtained through the hydrolysis of alkoxysilanes at low concentration, is less compact and more porous than silica; it is therefore no surprise that the silicagel nanoparticles show significantly different (and lower) values of Zeta potential both in an un-coated and in a polycation-coated. The different network topology would allow both for a better retention of counterions (a higher surface area would minimize their reciprocal repulsion) and for the adsorption of polycation also in the bulk of the nanoparticles. The latter would also permit GMMA to polymerize within the particles, increasing their size from the core and not solely from the shell. Correspondingly, one would expect the nanoparticles to increase in size homogeneously rather than being surrounded by a layer of external, softer material. Indeed AFM pictures seem to support this hypothesis, showing that the silicagel
nanoparticles show a virtually indistinguishable globular or “rocky” morphology before and after the very rapid polymerization (Figure 2-18).

Further investigation will be needed to confirm that GMMA polymerization does predominantly occur in the core of silicagel nanoparticles, but we are inclined to believe this phenomenon to be real.

Figure 2-18. Tapping mode AFM images of silicagel nanoparticles before and after ATRP of GMMA; no significant differences were recorded in the nanoparticle morphology. The curves in the white insert show the HRP activity test (pyrogallol test carried out on 0.1 mL of a 0.5% wt. nanoparticle dispersion in deionized water) performed on the DMF/water mixtures before the polymerization and after 25 minutes of polymerization, showing a significant retention of the enzymatic activity.

Finally, it is crucial that the polymerization conditions do not affect too detrimentally the enzymatic activity of horse radish peroxidase entrapped in the silicagel nanoparticles. The relatively high concentration of the monomer, which e.g. may act as a Michael-type acceptor for the nucleophilic groups (mostly primary amines) of the enzyme, or the large amount of DMF may indeed lead to denaturation of HRP. Samples were taken from the GMMA polymerization initiated by silicagel-pDMAEMA$_{40}$-pHEMA$_{40}$ nanoparticles in DMF/water 1:1 before the beginning and after 25 minutes of
polymerization, and were dialyzed to remove DMF and GMMA. The HRP activities of the resulting water dispersions were 0.37±0.10 and 0.20±0.08 U/mg of solid U/mL, respectively for t = 0 and 25 minutes; the comparison to the activity of the original nanoparticles (0.48±0.10 U/mg of solid) shows that, although a significant reduction was observed, a relevant fraction of the enzyme remained viable after the exposure to organic solvent and monomer and also during the successive polymerization.

2.5 Conclusions and perspective

In this work, ATRP was successfully applied to the derivatization of inorganic nanoparticles. First, we have optimized processes for the preparation of enzyme-containing silicagel nanoparticles and for their coating and stabilization with a polycationic layer. In the optimization phase, we have proved that the macroinitiator structure (degree of polymerization, ratio between positive charges and ATRP initiators) did not have any sound influence on the characteristics of the adsorption (zeta potential, agglomeration) on two different kinds of silica nanoparticles; this phase has therefore allowed a rational choice of the macroinitiator and of the conditions for its adsorption: high bromoester content, medium or low molecular weight and relatively low concentration for the macroinitiator, relatively high concentration for the nanoparticles.

We have also shown that the above described conditions did apply to silicagel nanoparticles too, and that both silica and silicagel nanoparticles can be easily purified via gel filtration, while enzymatic activity is substantially retained during both macroinitiator adsorption and gel filtration.

Finally, ATRP could be performed on the coated nanoparticles; the polymerization reached a rather rapid stop on silica nanoparticles, while rapidly proceeded to very high conversion in an apparently living fashion on silicagel nanoparticles, with a substantial retention of the activity of encapsulated enzymes. The difference between the polymerization on the two different substrates has been tentatively ascribed to the possibility of initiating both surface and bulk polymerization in silicagel nanoparticles.
It is noteworthy that the solvent power of the water/DMF mixtures used in the surface-initiated ATRP could allow the synthesis of a variety of different coatings, since ionic monomers (e.g. potassium 3-sulfopropylmethacrylate), thermosensitive ones (e.g. N,N-dimethylaminoethyl methacrylate, N-isopropylacrylamide), and even truly hydrophobic ones can be in principle polymerized in a controlled fashion in these solvents.

In terms of future perspectives that this study may open, the most important point is that enzyme-containing nanoparticles can be produced and functionalized with layers of organic polymers, which can provide “stealth” properties to the supported enzymes. This can open new possibilities for the application of pro-drug therapies: a bacterial enzyme can be protected by degradation, made non-immunogenic and possibly targeted by a “stealth” nanoparticle host, while being still active and therefore able to produce chemical conversions that are not possible for human enzymes. In this way a enzymatically activated pro-drug could be converted only at the location of nanoparticle accumulation, e.g. a tumoral site.

2.6 Appendix A – macroinitiator characterization

**Synthesis of the precursor polymer.** The protons of the CH$_3$ ($\alpha$) and CH$_2$ groups ($\beta$) of the main chain, and of the CH$_2$ ($\gamma$,\,$\delta$,\,$\varepsilon$,\,$\zeta$) and CH$_3$ groups ($\eta$) of the side chains present characteristic $^1$H-NMR resonances (Figure 2-19) that can be used to determine the molar ratio of the two monomers in the copolymers. The spectra were recorded in deuterated chloroform at $T = 25^\circ$C; a typical spectrum is reported in Figure 2-19.
Figure 2-19. $^1$H-NMR spectrum of a statistical copolymer DMAEMA$_{40}$-HEMA$_{40}$ showing resonance area of the protons selected for the monomer molar ratio determination.

If we define $P_1$ as the integral value of one proton of the side chain methyl groups of DMAEMA units (corresponding to 1/6 of the integral of protons $\eta$ depicted in Figure 2-16), and $P_2$ as the integral value of one proton of the methylene protons of HEMA units (corresponding to 1/2 of the integral of protons $\varepsilon$ depicted in Figure 2-19), then $P_1 =$ and $P_2 =$ . From the $P_1 / P_2$ ratio it is then possible to calculate the monomer molar ratios, which are approximately equal to 3:1 and 1:1 in agreement with their theoretical values.
The GPC analysis was performed dissolving the samples in DMF. The elution profiles of all the copolymers presented a monomodal and rather narrow gaussian distribution and the absence of tailing peaks indicated that no low molecular weight polymer is present (Figure 2-20); in order to determine molecular weight and polydispersity index, the GPC elution profiles were combined with a calibration curve using monodisperse poly(methyl methacrylate) standards. The number average molecular weights calculated from the GPC analysis are in fairly good accordance with the theoretical values: some discrepancy between the theoretical and GPC-derived values can be attributed to the difference in hydrodynamic volumes between the poly(methyl methacrylate) standards and the statistical copolymers.

**Introduction of initiator groups.** The solubility of the esterified copolymers in chloroform was not excellent, and the spectra were recorded in deuterated water at T = 25°C; a typical spectrum is reported in Figure 2-21.
Figure 2-21. $^1$H-NMR spectrum of an esterified pDMAEMA-HEMA copolymer showing the proton resonances selected for calculation of the degree of esterification.

Similarly to the previous case, defining $P_3$ as the integral value of one proton of the side chain methyl groups of esterified HEMA units (corresponding to $1/8$ of the integral of protons $\beta$ and $\theta$ depicted in Figure 2-21) and $P_4$ as the integral value of one proton of the main chain methyl groups of HEMA units (corresponding to $1/3$ of the integral of protons $\alpha$ depicted in Figure 2-21), one has $P_3 =$ and $P_4 =$. From the $P_3 / P_4$ ratio it is possible to calculate the degree of esterification for all the different copolymers. It is worth to point out that chemical shift values of the side chain methyl groups of DMAEMA units ($\eta$ and $\bar{\varepsilon}$) appear much higher than the ones in the polymer precursor; although a solvent effect would be expected there might be a substantial amount of protonated DMAEMA units in the polymer chain. $^1$H-NMR analysis indicated a degree of esterification of at least 95% with respect to the HEMA residues.
To determine the degree of quaternization, the spectra were recorded in deuterated water at $T = 25^\circ C$; a typical spectrum is reported in Figure 2-22.

**Figure 2-22.** $^1$H-NMR spectrum of a quaternized pDMAEMA-HEMA copolymer showing resonance area of the protons selected for the degree of quaternization calculation.

Similar to the previous cases, defining $P_5$ as the integral value of one proton of the side chain methyl groups of quaternized DMAEMA units (corresponding to 1/9 of the integral of protons $\eta$ depicted in Figure 2-22) and $P_6$ as the sum of the integral values of one proton of the side chain methyl groups of quaternized (corresponding to 1/9 of the integral of protons $\eta$ in Figure 2-22) and non-quaternized DMAEMA units (corresponding to 1/6 of the integral of protons $\chi$ in Figure 2-22), then $P_5 = \eta / 9$ and $P_6 = (\eta / 9) + (\chi / 6)$. From the $P_5 / P_6$ ratio it is possible to calculate the degree of quaternization for all the different copolymers. The $^1$H-NMR analysis indicated a degree of quaternization of about 99% of the DMAEMA residues.
# Appendix B – Optimization of macroinitiator adsorption

Table 2-3: \( \zeta \)-potential values of Silica nanoparticles as a function of SiO\(_2\) and polymer concentration.

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^a, ^b Polymer and Silica concentration (% wt) after adsorption.
Table 2-4: $\zeta$-potential values of Ludox NPs at different silica and polymer concentration after adsorption.

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$^a$, $^b$ Polymer and Silica concentration (% wt) after adsorption.
Table 2-5: Size values of Silicagel nanoparticles as a function of SiO₂ and polymer concentration.

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Table 2-6: ζ-potential values of Silicagel nanoparticles as a function of SiO₂ and polymer concentration.

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a, b Polymer and Silica concentration (% wt) after adsorption.

c Flocculation of the sample after adsorption.

d The scattering intensity was too low to be detected.

Size distributions of each macroinitiator adsorption onto commercial silica, silicagel and LUDOX® silica nanoparticles are summarized in the following tables:
Figure 2-23: Size distribution of a) SiO$_2$ NPs and b) LUDOX NPs (top, \(c = 0.1\) wt %, middle, \(c = 0.05\) wt %, bottom, \(c = 0.01\) wt %) with DMAEMA$_{18}$-HEMA$_6$ [\(c = 0.5\) wt % (solid line), 0.25 wt % (dash line), 0.125 wt % (dot line), 0.0625 wt % (dash dot line), 0.5 wt % (dash dot dot line)].
**Figure 2-24:** Size distribution of a) SiO$_2$ NPs and b) LUDOX NPs (top, $c = 0.1$ wt %, middle, $c = 0.05$ wt %, bottom, $c = 0.01$ wt %) with DMAEMA$_{12}$-HEMA$_{12}$ ($c = 0.5$ wt % (solid line), 0.25 wt % (dash line), 0.125 wt % (dot line), 0.0625 wt % (dash dot line), 0.5 wt % (dash dot dot line)).
Figure 2-25. Size distribution of SiO₂ NPs a) c = 0.1 wt %, b) c = 0.05 wt % and c) c = 0.01 wt % with DMAEMA₃⁶-HEMA₁₂ [c = 0.5 wt % (solid line), 0.25 wt % (dash line), 0.125 wt % (dot line), 0.0625 wt % (dash dot line), 0.5 wt % (dash dot dot line)].
Figure 2-26. Size distribution of a) SiO$_2$ NPs and b) LUDOX NPs (top, \( c = 0.1 \text{ wt } \% \), middle, \( c = 0.05 \text{ wt } \% \), bottom, \( c = 0.01 \text{ wt } \% \)) with DMAEMA$_{24}$-HEMA$_{24}$ [\( c = 0.5 \text{ wt } \% \) (solid line), 0.25 wt % (dash line), 0.125 wt % (dot line), 0.0625 wt % (dash dot line), 0.5 wt % (dash dot dot line)].
Figure 2-27. Size distribution of a) SiO$_2$ NPs and b) LUDOX NPs (top, $c = 0.1$ wt %, middle, $c = 0.05$ wt %, bottom, $c = 0.01$ wt %) with DMAEMA$_{60}$-HEMA$_{20}$ [$c = 0.5$ wt % (solid line), 0.25 wt % (dash line), 0.125 wt % (dot line), 0.0625 wt % (dash dot line), 0.5 wt % (dash dot dot line)].
Figure 2-28. Size distribution of a) SiO$_2$ NPs and b) LUDOX NPs (top, $c = 0.1$ wt %, middle, $c = 0.05$ wt %, bottom, $c = 0.01$ wt %) with DMAEMA$_{40}$-HEMA$_{40}$ [$c = 0.5$ wt % (solid line), 0.25 wt % (dash line), 0.125 wt % (dot line), 0.0625 wt % (dash dot line), 0.5 wt % (dash dot dot line)].
2.6 References

3. Atomic force microscopy characterization of nanostructured materials

3.1 Summary

Atomic force microscopy offers a number of attractive features, among them the ability to simultaneously study the morphology of nanostructured materials and evaluate their physical and mechanical properties.

In this section we have exploited this versatile scanning probe microscopy for the characterization of various nano-structured materials, ranging from soft nanostructured particles to flat polymer surfaces.

We have thus evaluated the morphology of soft polysaccharides nanoparticles such as chitosan/hyaluronic acid, the thickness and the physical properties of antifouling polymer surfaces based on glycerol monomethacrylate and the mechanical properties (elasticity) of thermally responsive polymer brushes based on N-isopropylacrylamide.

3.2 Introduction

The advent of atomic force spectroscopy marked the beginning of significant advancement in polymer chemistry, material science and biology toward high-resolution molecular-scale imaging in three quantified dimensions with the simultaneous measurements of one or more physical/mechanical properties.

The atomic force microscope (AFM, Nobel prize in 1986 for Binnig and Rohrer for the fundamental scanning tunneling microscope) was initially invented as an imaging tool for determining surface topographies at sub-nanometer resolution. In recent years, AFM has also been developed as a technique for micro/nano-manipulation and force spectroscopy of materials at the sub-micron scale or even at the single molecular level \(^1\), \(^2\). While conventional techniques for determining the mechanical properties of materials are based on direct manipulation and visual
observation, which cannot be easily applied for materials with sub-micron size, with AFM-based techniques nano-structured materials can be observed and manipulated.

The general principle of AFM is to scan a sharp tip over the surface of a sample while sensing the so-called near-field physical interactions between the tip and the sample (mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces, Casimir forces, solvation forces, etc.).

The sample is mounted on a piezoelectric scanner which ensures three-dimensional positioning with high accuracy. While the tip (or sample) is being scanned in the (x, y) directions, the force interacting between tip and specimen is monitored with picoNewton sensitivity. This force is measured by the deflection of a soft cantilever, typically made of silicon or silicon nitride with a tip radius of curvature on the order of nanometers, which is detected by a laser beam focused on the free end of the cantilever and reflected into a photodiode; thus, the resulting map of the area \( s = f(x,y) \) achieved by the sample scanning represents the topography of the sample.

![Scheme 3-1. Schematic representation of an atomic force microscope.](image)

The AFM can be operated in a number of modes, depending on the application. In general, possible imaging modes are divided into static (also called contact) modes and a variety of dynamic (or non-contact) modes where the cantilever is vibrated.
In the static mode operation, the static tip deflection is used as a feedback signal. Because the measurement of a static signal is prone to noise and drift, low stiffness cantilevers are used to boost the deflection signal. However, close to the surface of the sample, attractive forces can be quite strong, causing the tip to “snap-in” to the surface. Thus static mode AFM is almost always done in contact where the overall force is repulsive. Consequently, this technique is typically called 'contact mode'. In contact mode, the force between the tip and the surface is kept constant during scanning by maintaining a constant deflection.

In the dynamic mode, the cantilever is externally oscillated at or close to its fundamental resonance frequency or a harmonic. The oscillation amplitude, phase and resonance frequency are modified by tip-sample interaction forces; these changes in oscillation with respect to the external reference oscillation provide information about the sample's characteristics. Schemes for dynamic mode operation include frequency modulation and the more common amplitude modulation. In frequency modulation, changes in the oscillation frequency provide information about tip-sample interactions. Frequency can be measured with very high sensitivity and thus the frequency modulation mode allows for the use of very stiff cantilevers. Stiff cantilevers provide stability very close to the surface and, as a result, this technique was the first AFM technique to provide true atomic resolution in ultra-high vacuum conditions \[^3\].

In amplitude modulation, changes in the oscillation amplitude or phase provide the feedback signal for imaging. In amplitude modulation, changes in the phase of oscillation can be used to discriminate between different types of materials on the surface and it can be operated either in the non-contact or in the intermittent contact regime. In dynamic contact mode (also called intermittent contact or tapping mode) the cantilever is oscillated such that the separation distance between the cantilever tip and the sample surface is modulated, preventing thus the tip from sticking to the surface \[^4\].

In tapping mode, the cantilever is driven to oscillate up and down at near its resonance frequency by a small piezoelectric element mounted in the AFM tip holder. The amplitude of this oscillation is greater than 10 nm, typically 100 to 200 nm. Due to the interaction of forces acting on the cantilever when the tip comes close to the surface, near-field interactions cause the amplitude of this oscillation to decrease as the tip gets
closer to the sample. The resulting tapping mode image is therefore produced by imaging the force of the oscillating contacts of the tip with the sample surface. This is an improvement on conventional contact AFM, in which the cantilever just drags across the surface at constant force and can result in surface damage. Besides, if the sample surface is not homogeneous near-field interactions cause a shift not only in the amplitude of this oscillation but in its phase as well; distribution of the phase shift under scanning over the sample surface reflects distribution of the surface characteristics. Such mode of operation (Phase Imaging mode) is very useful for materials investigation to map variations in surface properties such as chemical composition, adhesion, friction, viscoelasticity, and perhaps other properties in comparison with surface topography.

In the non-contact mode, the tip of the cantilever does not contact the sample surface. The cantilever is instead oscillated at a frequency slightly above its resonance frequency where the amplitude of oscillation is typically a few nanometers (<10 nm). The van der Waals, which are strongest from 1 nm to 10 nm above the surface, or any other long range force which extends above the surface acts to decrease the resonance frequency of the cantilever. This decrease in resonance frequency combined with the feedback loop system maintains a constant oscillation amplitude or frequency by adjusting the average tip-to-sample distance.

Non-contact mode AFM reduces vertical applied forces and eliminates lateral forces, preventing thus tip or sample degradation effects that are sometimes observed after taking numerous scans with contact AFM. This makes non-contact AFM preferable to contact AFM for measuring soft materials, as biological samples. In the case of rigid samples, contact and non-contact images may look the same. However, if a few monolayers of adsorbed fluid are lying on the surface of a rigid sample, the images may look quite different. A cantilever tip operating in contact mode will penetrate the liquid layer to image the underlying surface, whereas in non-contact mode it will oscillate above the adsorbed fluid layer to image both the liquid and surface.

Another major application of AFM is force spectroscopy, the direct measurement of tip-sample interaction forces as a function of the distance between the tip and sample: the cantilever deflection is recorded as a function of the vertical displacement of the piezoelectric scanner, i.e. as the sample is pushed towards the tip and retracted. This results in a cantilever deflection (d) versus scanner displacement (z) curve, which can be
transformed into a force–distance curve by converting the cantilever deflection into a force (F) using Hooke’s law (F = -k × d, where k is the cantilever spring constant) and subtracting the deflection from the scanner displacement to obtain the distance (z-d). The point of contact (zero separation distance) is determined as the position of the vertical linear parts of the curve in the contact region.

Force-distance curves can be recorded either at single well defined locations of the (x, y) plane or at multiple locations to yield a so-called “force-volume image”. In doing so, spatially resolved maps of sample properties and molecular interactions can be produced. However, for quantitative force measurements, calibration of the actual spring constants of the cantilevers is necessary [5].

Problems with the technique include the common need for low stiffness cantilevers which tend to 'snap' to the surface. The snap-in can be reduced by measuring in liquids or by using stiffer cantilevers, but in the latter case a more sensitive deflection sensor is needed.

Many researchers have employed the AFM force spectroscopy in various ways to determine sample mechanical properties [6-9] such as elasticity (Young’s modulus), hardness, Hamaker constants, adhesion, and surface charge densities. These generally include nano-deformation tests, such as three-point bend tests and the more common nano-indentation [10], [11] where a sharp and rigid tip is used to probe the sample, recording applied forces and penetration depth.
Figure 3-2. Example of force curve from nanoindentation experiment showing adhesion between the cantilever and the sample surface.

The AFM has several advantages over the scanning electron microscope (SEM). Unlike the electron microscope which provides a two-dimensional projection or a two-dimensional image of a sample, the AFM provides a true three-dimensional surface profile with atomic resolution.

Additionally, samples viewed by AFM do not require any special treatments (such as metal/carbon coatings) that would irreversibly change or damage the sample. While an electron microscope needs an expensive vacuum environment for proper operation, most AFM modes can work perfectly well in ambient air or even a liquid environment. This makes it possible to study biological macromolecules and even living organisms.

In principle, AFM can provide higher resolution than SEM. It has been shown to give true atomic resolution in ultra-high vacuum (UHV) and, more recently, in liquid environments. High resolution AFM is comparable in resolution to scanning tunneling microscopy and transmission electron microscopy.

A disadvantage of AFM compared with the scanning electron microscope (SEM) is the image size. The SEM can image an area on the order of square millimeters with a depth of field on the order of millimeters. The AFM can only image a maximum height on the
order of 10-20 micrometers and a maximum scanning area of about 150×150 micrometers. But this is presently being improved, for instance by the use of parallel probes such as used by IBM’s "millipede" data storage concept.

Another inconvenience is that an incorrect choice of tip for the required resolution can lead to image artifacts. Traditionally the AFM could not scan images as fast as an SEM, requiring several minutes for a typical scan, while a SEM is capable of scanning at near real-time (although at relatively low quality) after the chamber is evacuated. The relatively slow rate of scanning during AFM imaging often leads to thermal drift in the image making the AFM microscope less suitable for measuring accurate distances between topographical features on the image although several methods were proposed to eliminate image distortions induced by thermal drift [12], [13].

AFM images can also be affected by hysteresis of the piezoelectric material [14] and cross-talk between the x, y, z axes that may require software enhancement and filtering. Such filtering could "flatten" out real topographical features. However, newer AFMs utilize closed-loop scanners, which practically eliminate these problems, or separated orthogonal scanners (as opposed to a single tube), which also serve to eliminate part of the cross-talk problems.

We have thereby focused on the application of such advanced microscopy technique in the imaging and force spectroscopy study of nano-structured systems, evaluating their morphological structures, elasticity (Young’s modulus) and adhesion forces.

3.3 Characterization of nano-structured materials

3.3.1 Methods

Each sample solution (20 µl) was placed on a freshly cleaved mica support and waited until complete evaporation. All AFM measurements were performed at room temperature using a Molecular Force Probe 3D AFM (MFP-3D, Asylum Research, Santa Barbara, CA) equipped with a 90 µm scanner. Silicon nitride cantilever (model NP-S20, Veeco, Santa Barbara, CA) with a nominal spring constant of 0.12 N/m and a
resonance frequency of 4 kHz was utilized in all measurements. The apparatus was allowed to equilibrate for 15 minutes prior to each measurement to reduce the thermal drift and afterwards images were collected using the contact or tapping imaging mode and a scan rate of 1 Hz.

**Nano-indentation.** AFM analysis was performed at room temperature in H₂O in order to avoid adhesion phenomena between the cantilever tip and the sample surface, using a Molecular Force Probe 3D AFM (MFP-3D, Asylum Research, Santa Barbara, CA) equipped with a 90 µm scanner. Silicon nitride cantilever (model NP-S20, Veeco, Santa Barbara, CA) with a nominal spring constant of 0.12 N/m and a resonance frequency of 4 kHz was utilized in all measurements. A Bio-Heater™ (Asylum Research) was employed to control the temperature for the measurements in water and the system was allowed to equilibrate for 15 minutes prior to each measurement to reduce the thermal drift. Measurements on mica were used to calibrate the deflection sensitivity of the instrument that is necessary to convert force-displacement measurements into force vs indentation dependences. In all force displacement measurements scan rate was 200 nm/s and indentation continued until a maximum level of compressive force of 8 nN. Spring constant (0.08 N/m) was measured using the thermal noise method [15]. The raw cantilever deflection versus probe displacement measurements have been converted into force-separation relations using the tip spring constant and the cantilever deflection sensitivities[16]. The force curves collected during the extension-retraction cycle were used for Young’s modulus calculations using only the initial slope of the extension part of the force-indentation curve.

The Young’s modulus can be calculated using the Hertzian model:

\[ F = \frac{4 E^* R^2}{3 (1 - \sigma^2)} \left(\frac{1}{\delta^2}\right) \]

Eq. 3-6

Where \( F \) is the load, \( \delta \) is the indentation, \( R \) is the tip radius, \( \sigma \) is the poisson ratio of the sample and \( E^* \) the effective modulus of a system tip-sample, which is calculated from the equation:
\[ \frac{1}{E^*} = \frac{1 - \sigma_{\text{tip}}^2}{E_{\text{tip}}} + \frac{1 - \sigma_{\text{sample}}^2}{E_{\text{sample}}} \]

Eq. 3-7

In which \(E_{\text{tip}}, \sigma_{\text{tip}}\) and \(E_{\text{sample}}, \sigma_{\text{sample}}\) are the Young’s modules and the Poisson ratios for the materials of tip and the sample, respectively; if the material of the tip is considerably harder than the sample the following equation is used \(^1\):

\[ \frac{1}{E^*} \approx \frac{1 - \sigma_{\text{sample}}^2}{E_{\text{sample}}} \]

Eq. 8-3

The Sneddon’s variation of the Hertz model is used for the case of cone tip of AFM cantilever \(^{15}\):

\[ F(h) = \frac{2}{\pi} \tan \alpha \left( \frac{E_{\text{sample}}}{1 - \sigma_{\text{sample}}^2} \right) h^2 \]

Eq. 3-9

Where \(\alpha\) is the half-opening angle of the AFM tip.

**Adhesion force.** All AFM measurements were performed in air at room temperature in contact mode using silicon nitride probes (model NP-S20, Veeco, Santa Barbara, CA) with a nominal spring constant of 0.12 N/m and a resonance frequency of 4kHz and allowing the system to equilibrate for 15 minutes prior to each measurement to reduce the thermal drift.

### 3.3.2 Chitosan/hyaluronic acid nanoparticles

One of the main interest of our group is the design of nano-carriers for payloads delivered in a biological environment and, specifically, of nanoparticles responsive to physico-chemical or biological stimuli that characterise the target environment \(^{18}\): for example, nanoparticles that undergo morphological transitions that modify release kinetics or uptake by cells in response to the presence of oxidants \(^{19}\).
Atomic force spectroscopy provides an ideal opportunity to study the morphology of nanoparticles and allows to investigate their shape, structural changes and stability. For example, contact mode AFM analysis of enzyme-containing polypyrrole nanoparticles [20] permitted to evaluate the shape, size and different flexibility of such particles deposited on silica and platinum substrates. Height and phase tapping mode-AFM were employed to examine topography of biocompatible hybrid poly(dimethysiloxane) nanoparticles in order to study their time-dependent surface morphologies and the intrinsic wetting behaviour [21].

Novel polysaccharidic nanostructures for drug delivery were also investigated by atomic force spectroscopy. Chitosan nanoparticles with sizes of 200-400 nm and a surface potential of 55-60 mV were achieved from ionic gelation between chitosan and pentasodium triphosphate (TPP); subsequently, chitosan-recombinant gene vaccine protein (RGVP) nanoparticles (potential Hepatitis B vaccine carriers) were prepared and their surface morphology analyzed by tapping mode AFM [22].

We have thus focused on the characterization of the nanoscopical structure of chitosan-based particles displaying hyaluronic acid (HA) on the surface [23]. Chitosan was dissolved in 4.6 mM HCl at concentrations 0.038%, 0.054%, 0.069%, 0.085% and 0.1% wt. adjusting the pH of the different solutions to 3, 4, 4.5 or 5 by the addition of appropriate volumes of NaOH 0.1 M. All solutions were sonicated for 40 min.

Solutions were filtered through a 0.22 μm pore size filter and, in order to remove any macroscopic material possibly present. The complexation was then carried out at 25°C and under magnetic agitation (750 rpm) for a duration of 30 min, followed by sonication for 40 min, leaving then the dispersion undisturbed for additional 16 h prior to any purification (ultrafiltration through 500 kDa molecular weight cut-off polyethersulphone (PES) membranes) or analysis. Dispersions with different nanoparticle content could be obtained by concentrating the dispersions during ultrafiltration and assessing their concentration by measuring the dry content after freeze drying.

Chitosan and TPP were used at relatively high dilution (0.035–0.093% and 0.007–0.017%, respectively) in order to avoid the formation of large aggregates. Having as a
target the preparation of nanoparticles with a high and positive $\zeta$-potential (for allowing the effective adsorption of a polyanion at a later stage), dimensions in the range 100–400 nm, and a long-term stability of both size and charge, we have conducted an optimization of the preparative process, monitoring the average value and the dispersity of size and $\zeta$-potential, as well as the stability of these values and of pH after complexation.

Seeking the best combinations of stability (of size, $\zeta$-potential and pH), high $\zeta$-potential and narrow size dispersity, we have therefore focused our attention on two samples characterized two samples of chitosan/TPP nanoparticles, characterized by a “small” (200–300 nm in deionised water) or a “large” (300–400 nm) size, and one sample of HA-coated chitosan/TPP nanoparticles were singled out for atomic force spectroscopy analysis; since the coating with hyaluronic acid is very likely not only to modify the nanoparticle surface, but also to increase their size, we have thus employed the “small” nanoparticles for coating experiments and the “large” ones as a reference system, similar in size and bulk composition to the coated ones, but displaying a chitosan-based surface. A graphical representation of the HA-coated chitosan/TPP nanoparticles is reported in figure 3-1.

![Figure 3-1. Graphical view of the HA-coated chitosan/TPP nanoparticles: TPP is present in the bulk of the nanoparticles bridging between positive charges of the chitosan chains. The negatively charged HA on the other hand binds chitosan on the surface of the nanoparticles, although a certain degree of diffusion in the bulk is possible. All nanoparticles were prepared by Dr A. Nasti.](image)

Figure 3-2. Graphical view of the HA-coated chitosan/TPP nanoparticles: TPP is present in the bulk of the nanoparticles bridging between positive charges of the chitosan chains. The negatively charged HA on the other hand binds chitosan on the surface of the nanoparticles, although a certain degree of diffusion in the bulk is possible. All nanoparticles were prepared by Dr A. Nasti.
AFM analysis of the three different kinds of nanoparticles was performed in tapping mode from dispersions in deionised water deposited on freshly cleaved mica surfaces; differently, in the measurement of HA-coated chitosan/TPP nanoparticles the anionic surface of mica was treated with a biological adhesive, poly-L-lysine, to enhance its adherence in order to avoid a possible removal of the specimen by the AFM tip during the scanning. The 3D topographic AFM micrographs (Fig. 3-3) show that chitosan nanoparticles were discernible as tall well-defined features while the analysis of the height scans along the median point allows to accurately calculating key dimensions as the height and the width of individual nanoparticles.
Figure 3-3. AFM analysis of the three different kinds of nanoparticles deposited on mica surfaces from dispersions in deionised water. The lower (left) or higher (centre) magnification pictures and the height scans along the median point of particles (right) highlight the larger dimensions of “large” and HA-coated nanoparticles compared to the “small” ones.

Figure 3-4. 3d AFM micrograph of “small” nanoparticles deposited on mica surfaces from dispersion in deionised water. The edge lengths are 2.5 μm.

A morphological comparison of the three different kinds of nanoparticles showed that:

A) The dimensional difference between “large”, “small” and HA- nanoparticles is real and not an artifact of the DLS analysis \(^{23}\). It is noteworthy that the HA-coated nanoparticles, although likely obtained through the agglomeration of few “small” ones, do not appear as clusters (Figure 3-3).

B) All nanoparticles are soft and flatten when deposited on a solid surface.
C) Both “Large” and HA-coated nanoparticles generally show some fracture lines as a consequence of drying (Figure 3-3), which suggests them to display a harder surface. “Small” nanoparticles appear to be always surrounded by a halo, which is fairly visible in height image (Figure 3-4) and more clearly in phase image (not shown), indicating it to be very soft and thin; we interpret it as a “fuzzy” corona composed by tethered chitosan chains. Consistently with this hypothesis, the halo disappears after coating.

3.3.3 Antifouling polymer surfaces

The surface chemistry, physics, and topography of a biomaterial are important parameters that influence the material properties\(^\text{[24-26]}\). Polymer brushes are an attractive means to control surface properties\(^\text{[27], [28]}\). Surface initiated ATRP has been widely used to fabricate a variety of polymer brushes that are of interest for a range of biomedical and bioanalytical applications and it offers precise control over many important parameters, including brush thickness, composition, and grafting density\(^\text{[29],[30]}\).

Polymer brushes based on poly(2-hydroxyethyl methacrylate) (PHEMA)\(^\text{[31]}\), poly(poly(ethylene glycol) methacrylate) (PPEGMA)\(^\text{[32-36]}\) and poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC)\(^\text{[37]}\), for example, can effectively suppress nonspecific protein adsorption and cell adhesion. AFM represents an extremely versatile technique to study the morphology, the structural changes in the polymer hydration state and bioadhesion of protein- and cell-repellent biocompatible surfaces. Besides, interactions between polymer-coated surfaces\(^\text{[38], [39]}\) and protein interactions with surfaces\(^\text{[38]}\) can be directly determined via force spectroscopy mode from the adhesion force curves.

In this section we have exploited atomic force spectroscopy for the characterization of conformal coatings with controlled thickness, firmly adsorbed/linkedd to a substrate producing protein-repellent (antifouling) films surfaces by using responsive polymers\(^\text{[49]}\).
We have first optimized a procedure of tissue culture polystyrene (TCPS) surface oxidation by studying the conditions for increasing the anionic charge density on its surface in order to allow the adsorption of cationic macroinitiators (quaternary ammonium and 1,2-bis(2-bromoisobutiryl) glycerol groups).

The resulting surfaces have then been used to initiate the ATRP polymerization of poly(glycerol monomethacrylate) (PGMMA) alone or in mixture with $N,N$-dimethylamino ethyl methacrylate (DMAEMA) producing films with controlled thickness. Subsequently, the films containing $N,N$-dimethylamino ethyl methacrylate (substantially hydrophobic at physiological pH) together with GMMA were singled out to study cell adhesion and spreading to comparable to TCPS (Figure 3-5).

**Figure 3-5.** Preparative steps for functionalized TCPS culture substrates: (a) the oxidized, anionically charged surface of a slide-on-flask set-up is coated with a polycationic macroinitiator (quaternary ammonium and 1,2-bis(2-bromoisobutiryl)glycerol groups in equal molar amounts; (b) the polymerization of gycerol monomethacrylate (GMMA), alone or in mixture with $N,N$-dimethylamino methacrylate (DMAEMA), produces films of controlled thickness; (c) after several washing steps cells are cultured in slide on flask set-up. All samples were prepared by E. Patrucco.
AFM morphological analysis of three different films grown on TCP surfaces (GMMA:DMAEMA at three different molar ratios) was performed both in air and in deionised water in order to evaluate either the polymer thickness either the influence of the swelling degree in a water environment on the different polymer composition.

The thickness of the films was thus evaluated by scratching them with a pipette tip, uncovering the harder TCPS substrate beneath, calculated from the analysis of the height scans along the resulting groove. Additionally, AFM data were compared with contact angle and infrared analysis.

The 3D topographic AFM micrographs (Fig. 3-6) highlight the different morphological features along the scratch between the TCPS surface and the polymer layer: for GMMA (both in air and in water) and the 50:50 sample (in water) this procedure allowed to peel off part of the film, revealing macroscopic areas of underlying TCPS surface. For the 50:50 molar ratio sample under air and the 70:30 sample (both in air and in water), the material was considerably harder and stickier and it was impossible to avoid the formation of debris along the scratches.

**Figure 3-6.** Contact mode AFM scans for the three polymer samples grown on TCPS slides in air (top) and under deionized water at 25°C.
AFM analysis showed that poly(GMMA) formed a layer of $210 \text{ nm} \pm 30 \text{ nm}$ in a dry state and $370 \text{ nm} \pm 50 \text{ nm}$ in a water-swollen state (deionized water); the large swelling degree, in agreement with the low value of the water contact angle (table 3-1), confirms poly(GMMA) to be very hydrophilic and extensively swollen in a water environment. Brushes of poly(GMMA) from surface initiated polymerization on aminated silicon wafers using similar polymerization conditions (1:1 v/v methanol/water solvent mixture and a CuCl/CuBr$_2$ catalyst) showed a similar layer thickness (calculated from ellipsometric studies) of about 175 nm in a dry state $^{[50]}$.

**Table 3-1.** Characterization data for SI-ATRP products on TCPS substrates after 24 h polymerization.

<table>
<thead>
<tr>
<th>GMMA/DMAEMA ratio (monomer feed)</th>
<th>contact angle</th>
<th>absorbance at 1735 cm$^{-1}$ (a.u.)</th>
<th>thickness in air $^b$ (nm)</th>
<th>thickness in water $^b$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>13 $\pm$ 4°</td>
<td>1</td>
<td>210 $\pm$ 20</td>
<td>370 $\pm$ 30 (1.76×) $^d$</td>
</tr>
<tr>
<td>70:30</td>
<td>48 $\pm$ 3°</td>
<td>1.33</td>
<td>260 $\pm$ 20 (1.24×) $^c$</td>
<td>290 $\pm$ 20 (1.12×) $^d$</td>
</tr>
<tr>
<td>50:50</td>
<td>90 $\pm$ 4°</td>
<td>2.85</td>
<td>430 $\pm$ 10 (2.05×) $^c$</td>
<td>490 $\pm$ 30 (1.14×) $^d$</td>
</tr>
</tbody>
</table>

$^a$ The intensity is normalized to 1 for the poly(GMMA) to facilitate the comparison.  
$^b$ Average and standard deviation on five points.  
$^c$ In brackets the increase compared to the poly(GMMA) sample.  
$^d$ In brackets the increase compared to the samples in air.

Differently, DMAEMA/GMMA polymers are expected to provide surface films that are substantially more hydrophobic and marginally positively charged. The SI-ATRP of 70:30 and 50:50 monomer mixtures from macroinitiator-containing surfaces provided thicker films compared to the homopolymerization of GMMA (table 3-1); additionally, the smaller swelling degree in aqueous medium, in agreement with higher values of the water contact angle, confirms the more hydrophobic degree of the films. Finally the film thickness measured via AFM on dry samples correlated reasonably well with IR data (table 3-1), which showed the intensity of the carbonyl stretching band to be strongly enhanced by the presence of DMAEMA. More detailed surface properties
information, such as elasticity and adhesion forces, would require AFM force spectroscopy analysis.

3.3.4 Thermally-responsive polymer surfaces

One of the main goals of our group is the design of a versatile method for preparing thermoresponsive surfaces that would provide a number of benefits in the contexts of smart surfaces [51], advanced membranes [52], microfluidics, cell sorting or cell harvesting [53].

Thermoresponsive polymers exhibit a temperature-dependent solution and surface behavior. Slight changes in the environment, for example, heating above the lower critical solution temperature (LCST), cause dramatic changes in the polymer hydration state and bioadhesion. Polymers such as poly(N-isopropylacrylamide) (poly(NIPAM)), poly(N-vinylcaprolactam), and poly(N-isopropylmethacrylamide) together with their associated copolymers are responsive to external stimuli such as pH and temperature. These materials have received a great deal of attention due to their unique properties and their potential for applications in bioengineering, nanotechnology, and future applications as constituents of biosensors and membranes [54-61]. The application of poly(NIPAM) microgels in controlled drug delivery is of particular research interest. Due to their open network structure and their ability to undergo large swelling and deswelling transitions, microgels, micrometer-sized particles of lightly cross-linked polymer, can incorporate small molecules, including certain drugs that can then be slowly released from the microgel interior. In light of this, many researchers have attempted to investigate the physical characteristics of poly(NIPAM) microgels.

Poly(NIPAM) exhibits a lower critical solution temperature (LCST) of about 32 °C in aqueous solution [62-63] and this property has been exploited in a number of applications, such as in drug uptake and release systems [64-67], rheological control [68], surface coatings [69],[70], water cleanup [71] and removal of water from biodiesel fuel [72]. Heating a solution of poly(NIPAM) changes the conformation of the polymer chain from an expanded coil to a collapsed globular structure as the solvation changes from good to
poor. Macroscopically, this can be observed as a turbid solution above the LCST which rapidly clears on cooling. This reversible transition is thought to be due to the negative entropy of mixing associated with the loss of hydrophobic interactions between the isopropyl groups of the polymer and water upon heating \([73]\). This behavior of poly(NIPAAm) has been well documented using a range of techniques, such as turbidimetry \([74-76]\), fluorescence studies \([77-78]\), light scattering \([71, 76, 79, 80]\), nuclear magnetic resonance \([81, 82]\) and small-angle neutron scattering \([76]\). Poly(NIPAAm) can be synthesized in various forms, including chains in solution, cross-linked gels, or poly(NIPAAm) microgel particles, depending upon the synthetic method used \([73]\). For example, gel beads can be created via classical precipitation polymerization or emulsion polymerization. The creation of a microgel particle during free radical polymerization involves the growth and collapse of poly(NIPAAm) chains when a critical chain length is reached. The collapsed poly(NIPAAm) chains serve as precursor particles for microgel formation. Generally, to form poly(NIPAAm) chains in solutions, techniques such as free radical initiation in organic solutions, redox initiation in aqueous medium, ionic initiators and radiation of aqueous medium \([73]\) are used. However, NIPAAm can undergo hydrogen-bridging with the amide groups of proteins, rendering NIPAAm likely not truly bioinert and thus prone to distort the results of certain cell assays. For the investigation of the surface transitions and structural changes triggered by the temperature shift, a sensitive analytic method is required. Atomic force microscopy is one of the most powerful current techniques for the characterization of such thermoresponsive polymer surfaces \([73-76]\) and has the unique advantage to perform a surface analysis of the physical/mechanical properties and the visualisation of important structural features simultaneously. Specifically, force curves measurements have emerged as a versatile tool to study surface forces \([77]\); the different surface properties (hydration state) of the polymer chains above and below the LCST result in different interactions between the surface and probe; below the LCST, at 25 °C, repulsive forces dominate the interaction; above the LCST, at 37 °C, attractive forces appear and stronger adhesion forces between surface and probe are detected. We have thus polarized our attention on the study of thermally-responsive surfaces prepared by grafting N-isopropylacrylamide (NIPAAm) via surface-initiated atom transfer radical polymerization (all NIPAAm samples were prepared by Dr Ruixue Liu).
from a cationic macroinitiator (MI) (ratio of reagents: [NIPAAm]:[MI]:[CuBr]:[N,N’,N”,N”'-Pentamethyldiethylenetriamine] = n:1:8:8) that was adsorbed onto a range of anionic substrates (mica, glass, quartz and high surface area carbon foam). The thermally-responsive PNIPAm layers were characterised in detail at 25 °C and 50 °C using atomic force microscopy to investigate their morphological differences; the layer thicknesses were also estimated by scratching the samples with a scalpel and measuring the height differences between untouched layer and scratched area.

Besides, the influence of temperature on the elasticity (Young’s modulus) and surface interactions between the polymer films and an AFM probe were also evaluated by nano-indentation experiments.

The PNIPAm films were analysed by contact mode AFM were prepared from a mica substrate using [M]₀/[MI]₀ values of 500 and 1000. AFM data are shown in Figure 3-7 and Table 3-2. At 25°C (Fig. 1a and b) both polymer layers exhibited a thickness of about 110 nm (light grey traces in Fig. 1e and f), while at 50 °C, (Fig. 1c and d) i.e. at a temperature well above the LCST for PNIPAm (ca. 32 °C), they had a thickness of 55–60 nm (Table 3-2) and a higher roughness than at 25 °C (red traces in Fig. 1e and f).

Both surfaces change from a low-temperature, swollen, state to a high temperature, collapsed, one. These data are indications of the success of the SI-ATRP approach used here for preparing thermally responsive surfaces. However, the data also show that the large difference in the [M]₀/[MI]₀ ratio appeared to have had relatively little influence on the average film thickness (as measured by AFM) and therefore on chain dimensions too. The true layer thicknesses for PNIPAm layers at temperatures less than the LCST should be larger than that measured by contact mode AFM measurements because of the small, but significant, compressive force applied. Indeed, there is some evidence for this below from the nano-indentation study. This effect is less significant for the collapsed PNIPAm layers. Nevertheless, these data are indications that the films may have suffered from a loss of living character/transfer to solution at some point of the polymerisation.
Figure 3-7. Contact mode AFM images and height traces obtained in water for PNIPAm grafted onto mica surfaces using $[M]/[MI]_0 = 500$ ((a), (c) and (e)) and 1000 ((b), (d) and (f)). The edge lengths are 10 μm. The typical height traces correspond to the height profile of a horizontal line passing at 5 μm from the front of the pictures. All samples were prepared by Dr R. Liu.
Table 3-2. AFM characterisation data.

<table>
<thead>
<tr>
<th>[M]/[MI]₀</th>
<th>Medium</th>
<th>Temp/°C</th>
<th>Thickness c</th>
<th>Ra a</th>
<th>Average peak to-peak distance c</th>
<th>E/kPa</th>
<th>Adhesion force/nN</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>Water</td>
<td>25</td>
<td>110</td>
<td>7.0</td>
<td>360 ± 160</td>
<td>51 ± 7</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>50</td>
<td>55</td>
<td>8.0</td>
<td>190 ± 50</td>
<td>3820 ± 1620</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Air c</td>
<td>50</td>
<td></td>
<td></td>
<td>-</td>
<td>4.0 ± 1.1</td>
<td>Nil</td>
</tr>
<tr>
<td>1000</td>
<td>Water</td>
<td>25</td>
<td>110</td>
<td>7.0</td>
<td>560 ± 260</td>
<td>48 ± 8</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>50</td>
<td>60</td>
<td>13.0</td>
<td>180 ± 100</td>
<td>1150 ± 160</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Air c</td>
<td>50</td>
<td></td>
<td></td>
<td>-</td>
<td>14.4 ± 1.9</td>
<td>Nil</td>
</tr>
</tbody>
</table>

a Line-average roughness.

b Values obtained taking the contact point of the force curves at a distance corresponding to the thickness of the film (see text).

c The values for the thickness (nm), Ra (nm) and average peak-to-peak difference (nm) were identical to those measured in water at 50 °C.

Scrutiny of the AFM surface profiles at 50 °C (Fig. 1e and f) shows some morphological differences between the two layers. Although both PNIPAm layers have a higher roughness at 50 °C than at 25 °C (Table 3-2), at high-temperature the sample prepared using [M]/[MI]₀ = 500 is markedly less rough. Furthermore, it is also possible to recognise some form of regularity in the height and spacing of the surface features ("peaks"). This partially ordered distribution is substantially absent for the layer prepared with [M]/[MI]₀ = 1000, where there is a large dispersion in peak height and spacing, although most still have a relative distance of 100-300 nm from each other.

Although it is not possible to completely exclude that the peaks correspond to individual polymer chains, their large size leads us to interpret them as inter-chain aggregates, possibly formed as a consequence of the phase separation during the temperature increase. The higher aspect ratio and the lower order of PNIPAm prepared using [M]/[MI]₀ = 1000 could be explained with the slower kinetics of the conformational rearrangements for longer PNIPAm chains, whose brushes are likely to have remained entrapped in a relatively extended conformation during thermally-triggered collapse.

Moreover, differences in both segment density and chain length within the layer can also strongly affect vertical collapse processes [71]. More detailed interfacial structural
information would require neutron reflectivity data; however, that is beyond the scope of the present study.

Nano-indentation experiments (figure 3-8) were carried out using small indentations in order to use the Hertzian model and approximate the tip geometry to a sphere, thus approximating the sphere radius to the nominal radius of the tip (10 nm).

**Figure 3-8.** Force curves for the indentation of the PNIPAm surface films. Left: polymers in dehydrated state at 50 °C; the mica substrate is shown for reference. Right: Sample prepared with [M]/[MI]₀ = 500 in water at 25 °C (the other sample provides analogous curves). The two arrows indicate the regions where the Young’s modulus was calculated.

Nano-indentation interaction forces between the cantilever tip and the polymer layers did not reveal significant differences between the two materials (Fig. 3-8 and Table 3-2). At 50 °C (Fig. 3-6, left) both polymers presented a Young’s modulus in the range of few MPa. The film prepared using [M]/[MI]₀ = 1000 showed a relatively lower value than that prepared using a value of 500, which may be caused by the higher roughness of the material (Table 3-2) and the resulting worse contact with the AFM tip. At 25 °C (Fig. 3-8, right) the water-swollen polymer layers showed a not surprisingly much softer character. The nano-indentation tip encountered some resistance also at distances from the surface (>200 nm) considerably larger than the film thickness (110 nm) for both polymers. The material composing this soft layer showed a Young’s modulus of about 1 kPa. A much higher modulus (about 50 kPa) was recorded taking the contact point of the force curves at a distance corresponding to the thickness of the film (Table 3-2).
This finding appears to indicate a progressively harder “gel” extending deeply in the water phase rather than the presence of a well-defined film/water interface. This could possibly have a contribution from surface-entrapped polymer originating from polymer grown in solution.

Adhesion forces were determined from the retraction force curves (not shown) derived from the indentation of the PNIPAm surface films. The silicon nitride cantilever tip was approached onto the surface with a maximum load of 20 nN and then immediately pulled off: upon retraction, the tip keeps in contact with the surface until the cantilever force overcomes the adhesion force. The latter can be directly read from the resulting sharp jump as the tip is pulled out of contact with the surface.

The adhesion data obtained (Table 3-2) showed no significant adhesion force at 25 °C, ensuring a substantially non-adhesive character of the water-swollen layer. This is consistent with related AFM studies of PNIPAm layers which reported that the maximum attractive force was nearly zero below the LCST \(^{[72]}\). With polymer chains in a collapsed state (50 °C) but still in a water environment, both samples showed a comparable, although fairly low, adhesion force to the silicon nitride tip (2.0 nN), confirming that both surfaces are able to provide thermally switchable adhesion. We can therefore suggest that for both of these PNIPAm surfaces an increase in temperature to above the LCST changes the surface from relatively hydrophilic with no significant adhesion to one that is hydrophobic and adhesive. Consideration of the data presented above shows that the main difference between the two surfaces is the one prepared using the higher \([M]_0/[MI]_0\) value (of 1000) has a greater roughness at 50 °C.

A limited AFM study was also conducted for the films held at 50 °C and then dehydrated and measured in air (Table 3-2). This state represents the greatest hydrophobicity possible for the polymer chains. It must be noted that the removal of water did not significantly alter the sample morphology and that the higher roughness of the film prepared using \([M]_0/[MI]_0 = 1000\) was preserved. Under these conditions, while the film prepared using \([M]_0/[MI]_0 = 500\) still presented a low adhesion (4.0 nN), that prepared using \([M]_0/[MI]_0 = 1000\) exhibited a noticeable increase (14.4 nN), with a pull-out force roughly seven times larger than that displayed in water (2.0 nN). Although it is generally assumed that an increase in roughness decreases the intensity of adhesion forces due to a reduction in contact area \(^{[82]}\), this picture can be often reversed in the
case of nano-structured surfaces. See for example the adhesion of Gecko fingers and the development of Gecko-inspired adhesives \cite{83}. We are therefore inclined to ascribe the larger adhesion for the $[\text{MI}]_0/[[\text{MI}]]_0 = 1000$ film in air to a Gecko-like mechanism. The adhesion data recorded here are broadly in agreement with those reported for poly(N-isopropyl acrylamide) microgels \cite{84}.

### 3.5 Conclusion

In this work atomic force microscopy was successfully applied for determining either surface topographies at sub-nanometer resolution either physical and mechanical properties of nano-structured materials.

The morphological comparison of different kinds of novel polysaccharidic nanoparticles for drug delivery by AFM imaging proved the dimensional difference between “large”, “small” and HA-nanoparticles pointing out thus specific morphological features (fracture lines, presence of coronas) and their relative diameter. AFM was also exploited to study the morphology, the structural changes in the polymer hydration state of protein- and cell-repellent biocompatible surfaces. Brush thickness, degree of swelling and different topographical features of antifouling film surfaces using responsive polymers were thus directly achieved by this extremely versatile technique.

Finally we have also shown that with the cantilever tip of this instrument it was possible to determine the principal mechanical (Young’s modulus) and physical (adhesion force) properties of thermally-responsive polymer surfaces by force spectroscopy analysis (nanoindentation).
3.6 References

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4. Self-assembled peptide gels

4.1 Summary

In this study we demonstrate how different homogenization technique (vortex vs. manual or orbital agitation) can dramatically influence the mechanical properties and the fibrillar organization of self-assembled peptide gels.

The pH-induced gelation of Fmoc-diphenylalanine and of its mixtures with Fmoc-diglycine was used to show how a different agitation during gelling can cause differences up to one order of magnitude in shear modulus, which was measured in oscillatory rheology and in creep and recovery experiments. AFM imaging and nanoindentation were then employed to highlight clear morphological, mechanical and physico-chemical differences in the gel fibrillar elements (ribbons).

As additional results, we have also demonstrated that mixtures of the two peptides exhibited the highest moduli both at a macroscopic level and at the nano-scale.

![Figure 4-1. Chemical structures of fluorenylmethyloxycarbonyl-diphenylalanine (Fmoc-FF) and fluorenylmethyloxycarbonyl-diglycine (Fmoc-GG).]
4.2 Introduction

The last twenty years have witnessed a continuously raising interest in organized and long-scale self-assembling phenomena of low molecular weight organic compounds; although hybrid organic-inorganic structures have been extensively investigated too [1],[2], most studies have specifically focused on the preparation of organogels [3] and hydrogels [4], i.e. 3D-extended and solvent-rich materials where self-assembled organic compounds provide connectivity at a molecular level and elasticity at a material one. The applications of such materials span from food [5] to controlled delivery [6, 7] and tissue engineering [8-10].

As building blocks, peptides have attracted more interest than other organic molecules [11, 12], since their structures can be both biomimetic and bioactive. Indeed peptidic self-assembly is at the basis of a vast number of naturally-occurring structures, whose biological activity depends on the morphological details of the aggregation process [13]; possibly the most popular example of self-assembled morphology is the aggregation of peptides/proteins in twisted β-sheets, which is known to be at the basis of the in vivo formation of amyloid fibrils and, at a larger scale, of plaques typical of dementia pathologies such as Alzheimer’s [14] of Creutzfeldt-Jakob [15] diseases. The field of amyloid-mimicking and fibrillar peptides has been authoritatively reviewed by Hamley [16]. Other biomimetic morphologies have been widely studied too, e.g. coiled coils [17], collagen-like [18] or elastin-like [19] structures and β-sheets from complex molecular architectures, such as hairpin peptides [20, 21]. It is also worth mentioning that peptides have also been used as functional groups for directing the self-assembly of polymer conjugates [22-25].

Although the attractive interactions between oppositely charged aminoacidic residues in a peptide can be used as the main driving force presiding to its self-assembly [26], the aggregation of hydrophobic patches is more commonly employed and may induce gelation also in case of mainly repulsive electrostatic interactions [27, 28]. A large number of peptidic or peptidomimetic amphiphilic structures have thus been studied [29], e.g.
with hairpin architecture \cite{20,30}, with alternated hydrophobic and charged residues \cite{26,31}, with terminal hydrophobic groups, such as Fmoc \cite{32-39}, etc.

Even the most favourable properties as biomaterials, however, would be useless in the absence of the possibility to control them. Therefore, a number of physical and chemical triggers have been used to switch on and/or off the self-assembly capacity of peptidic substrates, or at least to modulate it \cite{40}. Ionic strength \cite{41,42}, pH \cite{43,44}, temperature, enzymatic conversions \cite{45,46} to induce or remove the self-assembly conditions. Two points are specifically noteworthy: A) as it happens for amyloid proteins and amyloid-like peptides \cite{47} self-assembly may be substantially irreversible, but changes in pH and ionic strength may lead to the solubilisation of the smaller aggregates \cite{48}. Details of the material morphology may therefore influence the (ir)reversibility of gelation and the properties of the material. B) Aggregation and formation of 3D matrices are often rapid processes and a certain degree of kinetic control over the material morphology is not unlikely.

In the present study, we have focused on an important but often neglected detail of the preparative procedure, i.e. the method utilized to agitate the sample during a pH-triggered gelation, influences both morphology and mechanical properties of the peptidic gel. Specifically, we have employed two hydrophobically terminated dipeptides, Fmoc-diphenylalanine (FF) and Fmoc-diglycine (GG), individually or in mixture with different weight ratios, studying the rheological properties of the gels and their nano-scale morphology.

Various researchers have demonstrated that very short peptide sequences, containing just two or three amino acids with aromatic functionality (either provided by amino acid side chains, or by appended aromatic ligands) can form self-supporting nanostructured hydrogels \cite{49-57}. In these systems, aromatic interactions as well as hydrogen bonding play key roles. Variations in chemical structure (i.e. changes in amino acid sequence) have significant effects on the properties of the resulting gels. Peptides modified with aromatic groups such as 9-fluorenylmethoxycarbonyl (Fmoc), can thus self-assemble into hydrogels. These hydrogels have some similarities to extracellular matrices due to their high hydration, relative stiffness and nanofibrous architecture. Jayawarna et al \cite{58}
demonstrated that Fmoc-diphenylalanine (Fmoc-FF) provides a suitable matrix for two-dimensional (2D) or three-dimensional (3D) culture and how the introduction of chemical functionality into Fmoc-peptide scaffolds may provide gels with tunable chemical and mechanical properties for in vitro cell culture.

The aggregation of FF has also been recently demonstrated to be strongly pH-dependent \(^{[59]}\), with the formation of fibrils based on antiparallel \(\beta\)-sheets at high pH (9-10), lateral association of the fibrils at neutral or mildly basic pH (6-9) and final precipitation at mildly acidic pH (5-6). Here we have investigated whether this picture of thermodynamic control can be further complicated by kinetic effects, in our case the method of mixing.

### 4.3 Experimental section

#### 4.3.1 Peptide preparation and gel formation experiments

Hydrogels of Fmoc-diphenylalanine (FF) with Fmoc-GG were prepared in two ways: by utilizing a vortex mixer (Vortex-Genie® 2, Scientific Industries, Inc) and by hand or orbital shaking (Rotamax 120 orbital shaker, Heidolph). Fmoc-FF (MW = 535 g mol\(^{-1}\)) and Fmoc-GG (MW = 354) were purchased from BACHEM (Merseyside, UK); 21.4 mg of the pure peptides or of their mixtures in different weight ratios were placed in a 10 mL vial (diameter of 23.1mm and height of 46.6 mm); 2 mL of distilled water (Gibco®, UK) were added to obtain a 20mM total concentration of Fmoc-FF, and variable molar concentrations for the other formulations (e.g. 30 mM for Fmoc-GG, 25 mM of peptides for the 50% wt. mixture). Finally, 110 \(\mu\)L of 0.5 M sodium hydroxide was added to the aqueous suspensions of peptides until pH 10.5 was reached.

**“High-shear” (vortex) preparation.** The samples were vortexed for two minutes at roughly 1500-2000 rpm for 1 minute to fully dissolve the peptides; 0.1 M hydrochloric acid was then added to bring the pH to 6 while vortexing for 1 minute.

**“Low shear” (hand shaking”) preparation.** The samples were agitated for 2 minutes by hand at 3-5 strikes/second or placing them horizontally in an orbital shaker (200 rpm,
shaking orbit 20 mm); the two methods provide undistinguishable results. 0.1 M hydrochloric acid was then added to bring the pH to 6 and the samples were agitated for 2 minutes in identical manner.

After pH adjustment and final agitation, all gels were then incubated at 37°C for 12 hours to form firm gels. The final concentration of the peptides in the gel is about 1% wt.

4.3.2 Physico-chemical characterization

Rheology. A Gemini Advanced Rheometer (Malvern, UK) was used to perform all oscillatory and creep tests on the peptide gels, using a parallel plate geometry (upper plate: 25 mm diameter) and a gap of 100 µm. All measurements were performed in triplicate.

Appropriate amounts of gelled peptides were applied onto the lower plate to completely cover the measured area; the upper plate was then lowered and a pre-shear (10 Pa) was applied for 30 seconds to homogenize the samples. Oscillatory measurements were then run at a temperature of 25°C and a frequency of 1 Hz in controlled strain mode.

Creep and recovery tests were performed applying a variable stress (10-500 Pa, always within the viscoelastic region of the materials), and recording the strain as a function of time for 300 seconds; the stress was then removed and the strain was again recorded for additional 300 seconds. The compliance data were then fitted by a model derived from the standard linear solid (Zener) model, consisting of three elements in series: a spring, a Kelvin-Voigt element (spring and dashpot in parallel) and a dashpot. This model allows to separately take into account phenomena of purely elastic response, of viscoelastic response and of purely viscous flow. Correspondingly, the compliance can be described by the equation \[ J = J_0 + J_r (1 - e^{-t/\tau}) + \frac{t}{\eta} \] where \( J_0 \) is the instantaneous compliance (also known as glassy compliance), \( J_r \) is the retarded compliance which is equal to the inverse of the shear modulus \( G \), \( \tau \) is the retardation time associated with the
Voigt element and $\eta$ is the Newtonian (steady-state) viscosity. A more detailed explanation of the model is provided in the Electronic Supplementary Information.

**AFM analysis.** All AFM measurements were performed at room temperature using a Molecular Force Probe 3D AFM (MFP-3D, Asylum Research, Santa Barbara, CA) equipped with a 90 $\mu$m scanner. Silicon nitride cantilever (model NP-S20, Veeco, Santa Barbara, CA) with a nominal spring constant of 0.12 N/m and a resonance frequency of 4 kHz was utilized in all measurements. The apparatus was allowed to equilibrate for 15 minutes prior to each measurement to reduce the thermal drift Imaging and nanoindentation were performed as previously described in other publications.$^{[45]}$

**Imaging.** A sample solution (20 $\mu$L, ~ 0.1% wt. i.e. 10-fold dilution compared to the original preparation) was placed on a freshly cleaved mica support; after complete evaporation, the and afterwards images were collected using the tapping mode at a resonance frequency of 4 kHz and a scan rate of 1 Hz.

**Nanoindentation** AFM analysis was performed in deionized H$_2$O at a temperature of 20°C in a Bio-Heater™ (Asylum Research) using 1% wt. gels; measurements in a water environment were necessary in order to avoid adhesion phenomena between the cantilever tip and the sample surface. Young’s modulus was calculated by nanoindentation measurements following the same protocol used for the other nano-structured materials (see section 3.3.1 in chapter 3).

**Adhesion force measurements.** The adhesion forces between the AFM tip and the sample surface were determined from the retraction force curves after the indentation of the peptide hydrogels in air and were used to evaluate the surface energy of the samples. The cantilever tip was approached onto the surface and then pulled off: upon retraction, the tip keeps in contact with the surface until the cantilever force overcomes the adhesion force. The latter can be directly read from the sharp jump as the tip is pulled out of contact with the surface (adhesion force = difference between the force at zero and the minimum force$^{[50,51]}$). If we consider the adhesion force as the force employed to pull the tip out of contact with the surface, $F_{PO}$ (pull-off force), surface energy can be
calculated using the Johnson Kendall Roberts model:

\[ F_{PD} = -\frac{3}{2} \gamma \pi R \]  
Eq. 4-5

Where \( \gamma \) is the surface energy and R the tip radius.

### 4.4 Results and discussion

#### 4.4.1 Rheology

In the preparation of the peptidic gels we have initially adopted a mixing procedure based on the use of a vortex, which provides an efficient dispersion through the application of a high shear. Hereafter we will refer to these materials as “high shear” preparations.

First, it is worth noting that pure GG did not form gels; therefore, gel formation for FF/GG mixtures must be strongly dependent on the association of the aromatic residues.

All gels showed a linear viscoelastic region extending up to a strain value of about 0.02 (at a frequency of 1 Hz); at higher strain values most samples appear to be plastically deformed before undergoing macroscopic failure (Figure 4-2). With the exception of 100% GG, which did not gel, all peptide mixtures showed a qualitatively similar elastic behaviour, with a tan\( \delta \) value of about 0.17 - 0.3.
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Figure 4-2. Strain dependence of storage and loss moduli for some of the investigated gels with a constant ~1% wt. concentration and different Fmoc diphenylalanine (FF)/Fmoc diglycine (GG) weight ratios. $G'/G''$ values (and the corresponding error bars) are averages (and standard deviations) over three different preparations. Frequency = 1 Hz. All the graphs from “high shear” preparations, the “low shear” ones showed a very similar strain dependence, although different $G'$ and $G''$ absolute values. Measurements were performed by Dr W. Helen.

Figure 4-3. Dependence of storage and loss modulus and of tan $\delta$ on the composition of the hydrogel. Frequency = 1 Hz, strain = $10^{-3}$. Please note that pure GG does not provide gels. Measurements were performed by Dr W. Helen.
However, it was apparent (Figure 4-3) that FF-rich mixtures always exhibited considerably higher values of $G'$: at least 1 kPa vs. 0.1 – 1 kPa of GG-rich mixtures. Further, both $G'$ and $\tan \delta$ showed a significant dependence on composition when comparable quantities of the two peptides, and a maximum of $G'$ (17 kPa) and a minimum of $\tan \delta$ (0.17) were observed for a 1:1 (in weight) mixture of the two peptides, corresponding to a 3:2 GG/FF molar ratio.

The fluidodynamic details of the gelation process, however, may have an effect on the dynamics of the lateral aggregation of the peptide fibrils, leading to ribbons with a kinetically controlled later size due to convection characteristics of the mixing or to the mechanical instability of larger objects under high shear. In order to highlight the possible influence of agitation on the properties of the final materials, a few formulations (100% FF, 70% FF, 50% FF) were prepared through the same pH-dependent procedure, replacing the “high shear” vortex-based mixing with a more gentle manual or orbital agitation (undistinguishable results were obtained). Hereafter we will refer to these materials as “low shear” preparations.

The results of the oscillatory measurements were qualitatively in agreement with those of the “high shear” preparations, i.e. lowest $G'$ for 100% FF, intermediate for 70% FF and highest for 50% FF. However, all the “low shear” formulations showed clearly higher $G'$ and $G''$ values, and higher $\tan \delta$ values too. The increase was particularly impressive for the two mixed formulations, with a $G'$ values exceeding 100 kPa with 50% FF and 30 kPa with 70% FF.

Having in mind applications in tissue engineering, where cells may directly exert mechanical actions on materials, oscillatory measurements may not provide a complete picture of the mechanical behaviour of the gels; cells are indeed more likely to produce prolonged rather than oscillatory stresses. We have therefore investigated the linear viscoelastic behaviour of these gels by performing creep-recovery tests; typical creep-recovery curves are shown in Figure 4-4.
Figure 4-4. Creep-recovery curves for “high shear” (left) and “low shear” (right) gels with different peptide composition. The markedly higher compliance and the possibility of viscous flow of the “high shear” gels are apparent. Measurements were performed by Dr W. Helen.

With stresses ≥ 10 Pa, only the 70% FF gels showed a behaviour that could be fit with a Standard Linear Solid model (3-element Kelvin-Zener model: a Maxwell arm (dashpot + spring) connected in parallel to an elastic element (a second spring) [60]), while significant flow could be recorded for both 50% and 100 FF. We have therefore analyzed the creep and recovery data by the means of a modified Zener model, which contain a viscous element. The results of the fits are reported in Table 1, where the oscillatory rheology data for the same systems are provided for comparison.

The creep and recovery behaviour of “low shear” gels was investigated as a function of the applied stress in the range 5 – 500 Pa; with the exception of some of the data at σ = 500 Pa, no sound dependence on the applied stress could be highlighted and this ensures that for most of the applied stresses the systems fell within their linear viscoelastic regime. Isolated experiments at different stress values confirmed this also for “high shear” materials, which were therefore quantitatively studied only at a stress value of 10 Pa.

The instantaneous (glassy) compliance (\(J_0\)), which is responsible of the immediate mechanical response of the material, was always 2-4 times higher for the “high shear” samples. This indicates relatively higher propensity to instantaneous deformations, however, should not be used to extrapolate a higher free volume content for the “high
shear” gels, since their relaxation times (τ) were indistinguishable from those of “lo shear” ones, indicating a similar nature of the viscoelastic response and therefore excluding any sound difference in free volume. The shear modulus (G) was calculated as the reciprocal of the time-dependent component of compliance. Its values (Figure 4-4, left) clearly show that, as seen for G’ from oscillatory measurements, 50%FF always presented the highest modulus and 100% the lowest; however, the “high shear” preparations exhibited moduli about 5 times lower than those of “low shear” ones. In terms of steady state viscosity (η₀), which was added to take into account the plastic deformation of the samples, not surprisingly the samples with higher modulus exhibited also higher viscosities (Figure 4-5, right), and again “high shear” gels showed a clear difference from “low shear” ones.

![Shear modulus and steady state viscosity](image)

**Figure 4-5.** Shear modulus (G, left) and steady state viscosity (η₀) as a function of applied stress. The values are averages of the fitting results of both creep and recovery curves over three samples; error bars are not shown, but standard deviations are always below 10%. Measurements were performed by Dr W. Helen.
**Table 4-1. Summary of the mechanical characterization of the peptide hydrogels**

<table>
<thead>
<tr>
<th>% FF</th>
<th>Creep and recovery</th>
<th>Oscillatory rheology</th>
<th>Nanoindentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stress (Pa)</td>
<td>η&lt;sub&gt;0&lt;/sub&gt; (Pa·s)</td>
<td>τ (sec)</td>
</tr>
<tr>
<td>5</td>
<td>4.5·10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>24</td>
<td>1.0·10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<tr>
<td>10</td>
<td>6.7·10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>24</td>
<td>1.0·10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>7.7·10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>28</td>
<td>0.7·10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>500</td>
<td>4.0·10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>21</td>
<td>1.0·10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>1.0·10&lt;sup&gt;7&lt;/sup&gt;</td>
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<td>29</td>
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</tr>
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<td>1.0·10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>29</td>
<td>1.4·10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data in italics refer to the “high shear” samples. Rheology measurements were performed by Dr W. Helen.

<sup>b</sup> The results of the fits were averaged over three different samples and over both creep and recovery curves.

<sup>c</sup> Averages over two measures; oscillatory measurements performed at a strain of 10<sup>-3</sup> and a frequency of 1 Hz.

<sup>d</sup> The Young’s modulus was measured on samples swollen in deionized water at a temperature of 25°C; the surface energy was measured on dried samples (reference for surface energy: 2.35 nN/m for a mica surface).
4.4.2 Atomic Force Microscopy

In order to confirm the macroscopic behaviour of the peptide gels with understanding of the processes occurring at a fibrillar level, we have investigated the morphology of the various materials through AFM, further quantifying properties of the fibrils in nanoindentation.

The “high shear” samples (Figure 4-6) showed the contemporaneous presence of thin fibrils and larger ribbons characterized by a similar height (2.5 – 4 nm for all three peptide mixtures) and different lateral dimensions. Considerable thicker structures (in green in Figure 4-6, left half) are recognizable, which appear to arise from stacking or crossing of ribbons. The lateral dimensions of the fibrillar aggregates seem to increase with decreasing FF content and structures as wide as a few hundreds nanometers can be recognized in the 50% FF sample; this phenomenon was also qualitatively confirmed via SEM (see Appendix, Figure 4-11).

On the contrary, not only no sound increase in lateral dimensions was recorded on “low shear” samples with decreasing FF content, but the number of isolated fibrils was significantly lower than for “high shear” samples and so was the average lateral size of all ribbons (Figure 4-7). These differences were particularly evident for the 50% and 70% FF samples. A closer look to the 50% FF samples showed the presence of some form of regularity in the height profile of the tallest structures, both for “high shear” and “low shear” preparations (Figure 4-8). Following the profile along the direction of the tallest fibers, it was indeed possible to observe repetitive undulations, which were characterized by a relatively precise pitch in the case of “low shear” samples (210 ± 8 nm). These features suggest the possibility of a regular coiling of the ribbons around each other, e.g. a helical arrangement, to yield “super-ribbons”. Although a periodicity with a similar pitch was recognizable for “high shear” preparations too, this was more irregular and the height profile of the fibers displayed a number of double peaks that could be associated to interruptions of the coiling.

AFM has therefore highlighted two morphological differences, which are likely related to different modes of fibril aggregation during gelation: “high shear” samples appear to
be more prone to lateral aggregation, while “low shear” to a more regular form of entanglement.

The higher modulus of the “low shear” gels could then be due to the more regular interconnectivity between fibrils and ribbons, but differences in the internal physical chemistry and/or organization of the ribbons / “super-ribbons” could not be excluded. We have therefore performed nanoindentation experiments to evaluate the modulus and the surface energy of the materials at a level of individual fibers. In order to avoid any influence of the rigid substrate, the nanoindentation experiments were performed on thicker samples (a few hundreds microns of gels); there, the fiber morphology looked indistinguishable from those previously studied in tapping mode (see appendix, Figure 4-12). In these measurements we have selectively analyzed large ribbons, therefore the nanoindentation experiments provide an evaluation of the fiber bending modulus rather than of the Young’s modulus of the overall material; in Table 4-1, however, we keep the traditional symbol $E$ to refer to these values.
Figure 4-6. Tapping mode AFM scans for “high shear” samples. Left: 10*10 μm scans reporting the height profile along the median horizontal axis (dashed white line). Right: 1*1 μm scans reporting selected height profiles (in red).
Figure 4-7. Tapping mode AFM scans for “low shear” samples. *Left*: 10*10 μm scans reporting the height profile along the median horizontal axis (dashed white line). *Right*: 1*1 μm scans reporting selected height profiles (in red). The tallest ribbons often show signs of periodicity in their height profile and (see the bottom right picture) the overall shape may recall that of a multiple helix.
Figure 4-8. Tapping mode AFM scans for 50% FF samples produced under “high shear” (left) and “low shear” (right) conditions. Two areas per image are magnified to show “super-ribbons”, i.e. fibers with a more or less regular height periodicity. In the middle, the height scans recorded along the fiber axes.

Both for “high shear” and “low shear” materials, the fibers exhibited an increasing modulus with decreasing FF content (Figure 4-9, left), showing that the more rigid character of the materials is reflected in that of the mixed fibers; no dependence of the surface energy on composition was highlighted.

Rather surprising, however, the fibers of “low shear” materials revealed markedly lower moduli and surface energies (Figure 4-8, right) than those of the “high shear” ones. These differences are in both cases of about one order of magnitude, well beyond the experimental error (Table 4-1).
Figure 4-9. Left: typical indentation curves in water for “high shear” materials; the modulus clearly increases in the order 100%FF<70%FF<50%FF. Right: Comparison of the adhesion force curves for two compositions as the function of the mixing procedure. The much higher adhesion force (the “jump” upon detachment of the cantilever from the surface) of “high shear” materials is apparent.

The “high shear” materials therefore appear to be characterized by a larger amount of non-associated fibrils and by stiff and often rather wide ribbons, which expose a significant number of polar residues (higher surface energy) and show signs of irregular coiling. The “low shear” materials seem to have a lower fraction of unbound fibrils and softer but less polydisperse ribbons with a frequently regular internal (helical?) organization.

From the above, we hypothesize that the “low shear” conditions may have altered the mode of aggregation of ribbons, rather than that of fibrils. Coiled structures that minimize the surface energy through a more efficient use of polar residues would appear to have been favoured over lateral ribbon aggregation, which may on the contrary be predominant under the conditions of increased convection typical of “high shear” mixing.
4.5 Conclusions

The pH-induced gelation of Fmoc-dipeptides was shown to produce gels whose characteristics are markedly dependent on the preparative details, and specifically on an often neglected detail: the agitation procedure.

Accordingly, we have recorded differences up to one order of magnitude in the storage modulus measured through oscillatory rheology and in the shear modulus measured from creep and recovery experiments. These macroscopic differences are linked to the morphological organization of the gels, although the relationship seems rather complex: the fibers composing macroscopically stiffer (in shear deformation) gels appear to be softer (in bending deformation), to have a lower surface energy and to often present an internal, regular super-structure. Inter alia, this may suggest that the macroscopic modulus is possibly affected by the internal cohesion of the more elastically active elements (in this case large fibers made of assembled ribbons) rather than by their rigidity.

As a general take-home message, we wish to emphasize that the process of self-assembling may be heavily kinetically controlled, strongly affecting the macroscopic properties of the materials.

4.6 Appendix - Creep and recovery analysis

In the 3-element model Kelvin-Zener model the measured strain \( \varepsilon \) is expressed as a function of the applied (constant) stress \( \sigma_0 \) and of the elastic constants (moduli) of the two elastic elements, \( G_1 \) and \( G_2 \) (second elastic element, part of the Kelvin-Voigt unit), and of the retardation time arising from the viscous element of the Kelvin-Voigt unit:

\[
\varepsilon = \frac{\sigma_0}{G_1} + \frac{\sigma_0}{G_2} (1 - \exp(-t/\tau)) \quad \text{or, expressed in terms of the compliance } J, \quad J = \frac{1}{G_1} + \frac{1}{G_2} (1 - \exp(-t/\tau)) \ ; \text{this expression can be further rearranged in terms of the}
\]
overall shear modulus $G$ of the material and its instantaneous (glassy) compliance $J_0$:

$$J = J_0 + \frac{1}{G}(1 - \exp(-t/\tau)).$$

$1/G$ in this case can also be termed retarded compliance $J_r$.

The above description, however, can be applied only in case of complete recovery, where the maximum recorded compliance of the material under stress is $J = J_g + \frac{1}{G}$ and, once the removed the stress, the compliance returns more or less rapidly to zero. In our experiments, this was a reasonable approximation only for the 70% Fmoc FF gel.

In case of incomplete recovery (plastic deformation = flow), as it is clearly the case for both 50% and 100% Fmoc FF gels, a viscous loss term cannot be avoided; in this case, the residual compliance value after recovery, which we can call $J_{res}$, is related to a steady state viscosity $\eta_0$ of the materials: $\eta_0 = \frac{t_{appl}}{J_{res}}$.

Graphically, this can be represented by the insertion of a dashpot in series to a Zener model (Figure 4-10).

![Figure 4-10](image)

**Figure 4-10.** Graphical representations of the Zener model (left) and of the modified Zener model utilized in this study.
Using this modified model, the accord between experimental data and fits is excellent (Figure 4-11).

**Figure 4-11.** Creep curves fitted with the Standard Linear Solid (Zener) model (dotted lines) and the modified Zener model (solid lines). The experimental data are plotted as black squares (100% FF), open circles (70% FF) and open squares (50% FF). Only for 70% FF the two models provide both valid fits, while for the other two systems the modified Zener models is clearly superior, since it takes into account the viscous flow. Measurements were performed by Dr W. Helen.

NOTE: please realize that our approach is a significant approximation of a more realistic model where a distribution of relaxation (or retardation) times should be used; a function of distribution should therefore be introduced in the analytical expression of the compliance:

\[
J = J_0 + \frac{1}{G} \int_0^\infty f(\tau)(1 - \exp(-t/\tau))
\]
Figure 4-12. SEM images showing on “high shear” materials, showing a clear increase in lateral dimensions for the fibrillar aggregates (ribbons) with decreasing FF content.
Figure 4-13. Contact-mode scans of a 50% FF (left) and a 70% FF (right) “high shear” samples used for nanoindentation. These samples were obtained through the deposition of concentrated peptide dispersions (1% wt.). The different concentrations of the samples, however, did not seem to affect the morphology of the fibers; they appear substantially identical in lateral size and height to those obtained from the more dilute peptide dispersions used for imaging in tapping mode.
4.7 References


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5. Conclusions and perspectives

This work has demonstrated the feasibility of the preparation of well-defined nanoparticles containing active enzymes and displaying a synthetic, possibly “stealth” coating. The development of such materials can provide new powerful tools in medical bionanotechnology.

In terms of general perspectives, these novel nanoparticles represent promising vectors for pro-drug therapies, since they could allow long survival (“stealth” character) and targeted delivery to non-human enzymes that, therefore, can be possibly used for a local conversion of inactive pro-drugs into pharmacologically active compounds.

Further studies are required to progress this first proof of principle, e.g. by showing how the surface density and the chemical composition of polymer brushes may affect and modulate the cell uptake of and/or activation by the nano-structured delivery system in vitro and in vivo.

The use of enzyme-containing nanoparticles is envisaged for tumour therapy, therefore appropriate pro-drug / enzyme combinations will be considered. Since our proof of principle has involved horseradish peroxidase and this enzyme has already been used to convert indole-based pro-drugs, it is reasonable to suggest that (3-alkyl)indoles will be the first class of pro-drugs to evaluate.

At the same time, we have tackled the issue of imaging nanoparticles and characterizing nanoparticles at an individual level, extending our field of investigation to other nanostructured materials. We have polarized our attention on the use of Atomic Force Microscopy, which in recent years has evolved from a simple imaging technique to a multifunctional ‘lab-on-a-tip’, revolutionizing the nanotechnological research: AFM-based force spectroscopy is now used to study the mechanisms of molecular recognition and protein folding, or to probe the local elasticity, chemical groups and dynamics of receptor–ligand interactions in live cells, opening new avenues for medical diagnostics and environmental monitoring.

The main achievements of this work were a) to combine AFM to preparative work (vide supra), in order to evaluate purification and derivatization procedures, b) to use AFM
for the mechanical characterization of surface layers and nano-fibrillar materials. The next steps should involve an increase in the complexity of both the system analyzed and the information gathered, e.g. the use of AFM to probe the interactions of nanoparticles or other nano-structured materials with cells, measuring the force of interactions between cell surfaces and nanoparticles immobilized on AFM tips. This would allow, for example, to quantify the “stealth” character of nanoparticles and minimize the number of expensive in vitro and ethically questionable in vivo experiments.