Boronic-diol complexation as click reaction for bioconjugation purposes

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

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Abstract

The research presented in this thesis focuses on the study of the reaction between boronic acids and diols and its evaluation as a possible "click" reaction, possibly applicable in bioconjugation and drug delivery. A key feature of this reaction is its reversibility at acidic pH, which could allow the release of a diol-containing drug from a bioconjugate in the acidic environment of late endosome/lysosome, possibly after undergoing receptor mediated endocytosis.

Over the last two decades various studies have focused on the study of the conjugation of boronic acids to diols using Alizarin Red S as a fluorescence reporter. In this research we have presented an alternative method based on the batochromic shifts of Alizarin Red S absorbance; this method is particularly advantageous in complex systems with an elevated scattering, such as colloidal dispersions or for binding to complexed active compounds. We have therefore demonstrated that this method allows the determination of equilibrium constants between diols (e.g. catecholamines) and boronic acids.

We have also demonstrated that the method allows to follow the kinetics of enzymatic reactions involving catechols; in particular, we have focused on cytochrome P450-mediated reactions such as the conversion of estradiol to 2-hydroxyestradiol using CYP1A2, or the demethylation of 3-methoxytyramine to dopamine using CYP2D6.

Once we have established a reliable method for following this reaction on low molecular weight compounds, we have applied it to polymeric bioconjugates. Specifically, we have selected hyaluronic acid (HA) as a biocompatible and biodegradable polymeric backbone and produced derivatives containing boronic acids, catechols and dimethylated catechols (as negative controls). The resulting polymers where characterised via UV-Vis, $^1$H NMR and SLS, also qualitatively evaluating their cytotoxicity and enzymatic degradability. The conjugates with boronic acids showed the lowest cytotoxicity, and the highest degradability. The complexation of HA-boronic derivatives was then studied; using the same library of diols previously used with low molecular weight compounds, evaluating the effect of the presence of the polysaccharidic macromolecular chain.
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Dedication

To my Family and Friends
Acknowledgements

I would like to express my deepest appreciation and gratitude to Prof. Nicola Tirelli for offering me this PhD-position and guiding me through these four years of research and instilling in me this passion for science to a height I never imagined before. Nicola has moulded me from an engineer into a chemist and from a student into a scientist by guiding, teaching and helping me mature in both my personal and professional life as a teacher and as a friend.

I would also like to sincerely thank Dr. Francesco Cellesi for his valuable help, advice and support because without him this research would not have been complete. Francesco has helped me strengthen my skills and guided me through each step of the research.

This work would not have been possible without the help of many individuals. I would like to particularly thank Dr. Eman Abdeljaber who has assisted me in performing the cytotoxicity and degradability studies. I am grateful to all the members of the Laboratory of Polymers and Biomaterials who have spent these four years with me on a professional as well as a social scale.

I would like to thank all my friends in Manchester and beyond ~ “You know who you all are” with whom I have spent some of the most amazing times, from having coffee to partying, and for creating a social circle without which the last four years would have been a rocky ride.

Last but not least, I would like to thank my family who have raised, inspired, befriended, loved, guided, and supported me through the last quarter of a century at all times. Their life has always been and will always be an inspiration to my life.
## Abbreviations

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<tbody>
<tr>
<td>mg</td>
<td>$10^{-3}$ gram</td>
</tr>
<tr>
<td>mL</td>
<td>$10^{-3}$ litre</td>
</tr>
<tr>
<td>μL</td>
<td>$10^{-6}$ litre</td>
</tr>
<tr>
<td>mmol</td>
<td>$10^{-3}$ mol</td>
</tr>
<tr>
<td>picomol</td>
<td>$10^{-12}$ mol</td>
</tr>
<tr>
<td>μm</td>
<td>$10^{-6}$ meter</td>
</tr>
<tr>
<td>nm</td>
<td>$10^{-9}$ meter</td>
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<tr>
<td>M</td>
<td>mol.litre$^{-1}$</td>
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<tr>
<td>mM</td>
<td>$10^{-3}$ mol.litre$^{-1}$</td>
</tr>
<tr>
<td>μM</td>
<td>$10^{-6}$ mol.litre$^{-1}$</td>
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<tr>
<td>g/L</td>
<td>gram.litre$^{-1}$</td>
</tr>
<tr>
<td>mg/mL</td>
<td>$10^{-3}$ gram.$10^{-3}$ litre$^{-1}$</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton (g/mol)</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>$\overline{M_w}$</td>
<td>Weight Average Molecular Weight</td>
</tr>
<tr>
<td>$R_g$</td>
<td>Z-average radius of gyration</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
</tr>
<tr>
<td>$\lambda_{exc}$</td>
<td>Excitation wavelength</td>
</tr>
<tr>
<td>$\lambda_{em}$</td>
<td>Emission wavelength</td>
</tr>
<tr>
<td>UV-VIS</td>
<td>Ultra Violet-Visible (spectroscopy)</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeation and retention effect</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
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</tr>
<tr>
<td>ADAGEN</td>
<td>Poly(ethylene glycol)-bovine adenosine deaminase</td>
</tr>
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<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>PEGMA</td>
<td>Poly(ethylene glycol) methacrylate</td>
</tr>
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<td>DIC</td>
<td>N,N'-diisopropyl carbodiimide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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<td>RGD</td>
<td>Arginine-glycine-aspartate</td>
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<td>NIPAAm</td>
<td>N-isopropylacrylamide</td>
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<td>SH</td>
<td>Thiol</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>ATRP</td>
<td>Atom transfer radical polymerisation</td>
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<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
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<td>CuAAC</td>
<td>Copper-catalyzed azide-alkyne Huisgen 1,3-dipolar cycloaddition</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>ROP</td>
<td>Ring-opening polymerisation</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition-fragmentation chain transfer polymerisation</td>
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<tr>
<td>Boc</td>
<td><em>Tert</em>-butyloxy carbonyl</td>
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<tr>
<td>HEMA</td>
<td>Hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factors</td>
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<tr>
<td>NCA</td>
<td>N-carboxyanhydride</td>
</tr>
<tr>
<td>Ebib</td>
<td>Ethyl-2-bromo-2-methyl-propionate</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PEO-b-PGA</td>
<td>Poly(ethylene oxide)-block-poly(glycerol monoacrylate)</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilboestrol</td>
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<tr>
<td>BASE</td>
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<tr>
<td>pKₐ</td>
<td>Acid dissociation constant</td>
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<td>Kₐeq</td>
<td>Equilibrium binding constant</td>
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<tr>
<td>ARS</td>
<td>Alizarin red S</td>
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<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
</tr>
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<td>RHAMM</td>
<td>Receptor for HA mediated motility</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>IVD4</td>
<td>Intervertebral disc receptor</td>
</tr>
<tr>
<td>LEC</td>
<td>Liver sinusoidal endothelial cell receptor</td>
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<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<td>AcetylCoA</td>
<td>Acetyl coenzyme A</td>
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<td>HAS</td>
<td>Hyaluronan synthases</td>
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<td>UDP</td>
<td>Uridine 5’-diphosphate</td>
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<td>HYAL</td>
<td>Hyaluronidase</td>
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<td>TSG</td>
<td>Tumour suppressing gene</td>
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<td>SPAM</td>
<td>Sperm adhesion molecule</td>
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<td>DMF</td>
<td>Dimethylformamide</td>
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<td>N-hydroxysuccinimido diphenyl phosphate</td>
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<td>HYAFF</td>
<td>HA-esterified biomaterials</td>
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<td>ADH</td>
<td>Adipic acid dihydrazide</td>
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<td>DSS</td>
<td>Disuccinimidyl suberate</td>
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<td>Poly(vinyl alcohol)</td>
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<td>Poly(lactic-co-glycolic acid)</td>
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<td>Dihydroxymandelic acid</td>
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<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>Cytochrome</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>$K_{\text{trig}}$</td>
<td>Equilibrium constant for trigonal form</td>
</tr>
<tr>
<td>$K_{\text{tet}}$</td>
<td>Equilibrium constant for tetrahedral form</td>
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<td>APBA</td>
<td>3-aminophenyl boronic acid</td>
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<td>DI</td>
<td>Diol</td>
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<tr>
<td>$K_{\text{ARS}}$</td>
<td>Equilibrium constant for ARS-boronic reaction</td>
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<td>$K_{\text{Diol}}$</td>
<td>Equilibrium constant for boronic-diol reaction</td>
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<tr>
<td>$K_{\text{EXC}}$</td>
<td>Equilibrium constant for competitive binding reaction</td>
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<td>REDOX</td>
<td>Reduction-oxidation reaction</td>
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<td>MDMA</td>
<td>3,4-methylenedioxy-amphetamine</td>
</tr>
<tr>
<td>PBA</td>
<td>Phenylboronic acid</td>
</tr>
<tr>
<td><strong>Content</strong></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td><strong>DOPA</strong></td>
<td>Dopamine hydrochloride</td>
</tr>
<tr>
<td><strong>β-NADPH</strong></td>
<td>β-Nicotinamide adenine dinucleotide 2′-phosphate</td>
</tr>
<tr>
<td><strong>TCEP</strong></td>
<td>Tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td><strong>x_A</strong></td>
<td>Molar fraction of free ARS</td>
</tr>
<tr>
<td><strong>C_A</strong></td>
<td>Total concentration of ARS</td>
</tr>
<tr>
<td><strong>C_B</strong></td>
<td>Total concentration of boronic acid</td>
</tr>
<tr>
<td><strong>C_D</strong></td>
<td>Total concentration of diol</td>
</tr>
<tr>
<td><strong>ε</strong></td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td><strong>ω</strong></td>
<td>Parameter relating to band width</td>
</tr>
<tr>
<td><strong>K_1</strong></td>
<td>Equilibrium binding constant of ARS-boronic</td>
</tr>
<tr>
<td><strong>K_2</strong></td>
<td>Equilibrium binding constant of boronic acid-diol</td>
</tr>
<tr>
<td><strong>[S]</strong></td>
<td>Substrate concentration</td>
</tr>
<tr>
<td><strong>[S]_0</strong></td>
<td>Initial substrate concentration</td>
</tr>
<tr>
<td><strong>v</strong></td>
<td>Initial rate of reaction</td>
</tr>
<tr>
<td><strong>v_max</strong></td>
<td>Maximum rate of reaction</td>
</tr>
<tr>
<td><strong>I_o</strong></td>
<td>Total concentration of ARS in fluorescence model</td>
</tr>
<tr>
<td><strong>[L]</strong></td>
<td>Concentration of free boronic acid in fluorescence model</td>
</tr>
<tr>
<td><strong>HCl</strong></td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td><strong>SLS</strong></td>
<td>Static light scattering</td>
</tr>
<tr>
<td><strong>A_2</strong></td>
<td>Second virial coefficient</td>
</tr>
<tr>
<td><strong>dn/dc</strong></td>
<td>Refractive index dependence on solute concentration</td>
</tr>
<tr>
<td><strong>1/τ</strong></td>
<td>Reaction rate of enzymatic hydrolysis</td>
</tr>
<tr>
<td><strong>MTS</strong></td>
<td>(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)</td>
</tr>
<tr>
<td><strong>Gibco</strong></td>
<td>Glutamine</td>
</tr>
<tr>
<td><strong>FCS</strong></td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td><strong>EDTA</strong></td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td><strong>HA-3-APBA</strong></td>
<td>3-aminophenylboronic acid functionalised hyaluronic acid</td>
</tr>
<tr>
<td><strong>HA-Dopamine</strong></td>
<td>Dopamine functionalised hyaluronic acid</td>
</tr>
<tr>
<td><strong>HA-Veratrylamine</strong></td>
<td>Veratrylamine functionalised hyaluronic acid</td>
</tr>
<tr>
<td><strong>PEG-DOPA</strong></td>
<td>Dopamine functionalised poly(ethylene glycol)</td>
</tr>
<tr>
<td><strong>GlcA</strong></td>
<td>D-glucuronic acid</td>
</tr>
<tr>
<td><strong>GlcNAc</strong></td>
<td>N-acetyl-D-glucosamine</td>
</tr>
<tr>
<td><strong>η_{rel}(0)</strong></td>
<td>Initial viscosity</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$\eta_{\text{rel}}(\infty)$</td>
<td>Plateau viscosity</td>
</tr>
<tr>
<td>IC50</td>
<td>The half maximal inhibitory concentration</td>
</tr>
</tbody>
</table>
Chapter 1

1. Introduction and scope of the thesis

1.1 Bioconjugation

Bioconjugation is generally referred to as the formation of a chemical linkage between a biologically active component (protein, peptide or drug) and a polymer whereby the biologically active component provides the biofunctional properties, and the polymer component improves the stability, solubility and biocompatibility of the active component\(^1\textsuperscript{-5}\).

Bioconjugation with polymers provides several advantages to the bioavailability of the drug such as:

A) **Shielding effect.** Polymers usually protect the conjugated components from chemical and enzymatic degradation and can mask their antigenic sites, thereby possibly reducing their antigenic/immunogenic properties. For example, poly(ethylene glycol) (PEG) has a shielding effect on conjugated drugs from thermodynamic effects like temperature, pH and ionic strength, attack by immunoglobulins, degradation by enzymes, and adhesion to surfaces due to its high mobility, associated and conformational flexibility, and water-binding ability\(^2,5\textsuperscript{-8}\).

B) **Reduction in renal excretion.** The increase in volume following bioconjugation may allow reducing the elimination through kidneys, which is known to be size-sensitive, with a threshold generally assumed to correspond to the size of serum albumin. Since, however, the size depends not only on the molar mass, but also on the hydration of the bioconjugate, the threshold may occur at molar masses considerably lower than that of albumin. For example, PEG threshold occurs roughly at a molecular mass of one third of the protein one\(^2,5,9,10\).

C) **Enhanced permeability and retention.** Polymers can be conjugated with drugs to promote targeted delivery to sites characterised by increased capillary permeability, like tumoural or inflamed tissues. For example, this may increase the so-called Enhanced Permeation and Retention effect (EPR effect) that is based on the preferential accumulation of colloids in cancer tissues due to the higher permeability of blood capillaries along with a reduced lymphatic drainage of the tumour\(^2,4,5,10-13\).
D) **Receptor-mediated-endocytosis.** Macromolecular characteristics such as solubility, stability and recognition by receptors of the bioconjugates in blood are responsible for the drug’s entrance into the cell through adsorption or receptor-mediated-endocytosis and cleavability intracellularly. There are various linkages like acetals and boronic-diols which are acid-sensitive i.e. stable in blood at physiological pH and cleavable only intracellularly due to the acidic pH or enzymes of the endosomes and lysosomes\textsuperscript{2,6,13}.

Depending on the application, bioconjugates can be synthesised in various ways ranging from a head-to-tail design where the polymer serves as one block and the biomolecule as the other; to a comb shaped structure with biomolecules as side chains which grants high stability from denaturation due to its multi-point attachment sites; or high throughput dendrimeric structures which can either encapsulate functional molecules and isolate their active site or covalently link their functional peripheral groups to biomolecules\textsuperscript{1,2}. The various applications and techniques used for bioconjugation are mentioned in the following sections.

### 1.1.1 Applications of Bioconjugation

**Discovery of Biological Interactions.** An essential task for understanding biological processes is to discover and characterise natural ligands of biopolymers. Coupling biomolecules of interest with small synthetic molecules capable of ligand binding is one of the upcoming approaches for ligand discovery. These small synthetic molecules (fluorescent molecules\textsuperscript{2,14}, biotin\textsuperscript{15,16} or NMR probes\textsuperscript{15}) act as probes for ligand binding. Some popular examples of this approach are DNA microarrays\textsuperscript{15,17} and protein microarrays\textsuperscript{14,15} which enable a high-throughput screening of ligands.

**Biochemical Assays.** Bioconjugation of small molecules to biomolecules can serve as probes for biochemical analyses for example; in Förster resonance energy transfer (FRET) which generates signals sensitive to molecular conformational changes in the 1-10 nm range and can be used to characterise protein and RNA folding\textsuperscript{16}. FRET works by attachment of a pair of fluorescent molecules to different regions of a biomolecules whereby, one fluorophore serves as a donor and the other an acceptor of energy, and the
acceptor then emits radiation at its own frequency reporting the distance between the fluorophores.\textsuperscript{15, 18}

**Diagnostic Applications.** Qualitative and quantitative detection of analytes in clinical samples is crucial for the early diagnosis of disease. The most common biosensors used commercially consist of biomolecules attached to surfaces via robust bioconjugation linkages for example; glucose sensors\textsuperscript{8} (for monitoring glucose levels in diabetics) which record digital signals when glucose oxidase is converted to hydrogen peroxide by an immobilised enzyme.\textsuperscript{2, 8, 15, 19}

Some of the other biosensors use optical techniques such as surface plasmon resonance (SPR)\textsuperscript{15} to evaluate the binding of analytes to biomolecules immobilised on a surface.

**Imaging in vivo.** Magnetic resonance imaging (MRI)\textsuperscript{13, 15, 20}, radioimaging\textsuperscript{15, 21} and positron emission tomography (PET)\textsuperscript{15} are some of the most common techniques which are used for imaging in vivo. MRI requires contrast agents such as magnetite or Gd(III)\textsuperscript{15, 20} to be conjugated to antibodies while radioimaging uses isotopes of iodine (\textsuperscript{123}I and \textsuperscript{131}I) introduced into tyrosine residues of proteins\textsuperscript{15, 16}. PET is used to map normal human brain and heart function\textsuperscript{15} as well as for the clinical diagnosis of certain diffuse brain diseases such as the ones causing dementias\textsuperscript{15} by sensing the gamma rays emitted indirectly by a positron-emitting radionuclide (\textsuperscript{18}F fluoro group attached to glucose).

**Polymer-Protein/Drug linkages.** Bioconjugation of biodegradable polymers like poly(ethylene glycol) and hyaluronic acid with proteins and drugs is well known in literature. The linkage of proteins/drugs to these polymers is essential as it can provide desirable advantages such as reduced immunogenicity\textsuperscript{22, 23}, improved circulating half-life in vivo\textsuperscript{2}, enhanced water solubility\textsuperscript{15}, enhanced proteolytic resistance\textsuperscript{2}, reduced toxicity\textsuperscript{22} and improved thermal and mechanical stability\textsuperscript{23}.

**Industrial Applications.** Immobilised enzymes are widely used as industrial catalysts in the pharmaceutical, food and chemical industry\textsuperscript{8}. In the food industry they are used in the isomerisation of fumeric acid to malic acid\textsuperscript{15}. In the pharmaceutical industry they are used for synthesis of drugs like 6-aminopenicillanic acid which uses immobilised
penicillin amidase and in the chemical industry for preparation of acrylamide from acrylonitrile\textsuperscript{2,15}.

### 1.1.2 Bioconjugation Techniques

#### A) Grafting to using natural functionalities

i) **Amine conjugation (PEGylation).** One of the oldest and most common bioconjugation techniques is the reaction between the activated hydroxyl group of PEG chains and primary amines from proteins\textsuperscript{2,24,25}. Functionalisation by alkylation which maintains the positive charge of the protein at physiological pH and acylation which involves loss of charge at the conjugation site is possible due to the high numbers of primary amines present on the surface of proteins in both lysine residues and N-terminus\textsuperscript{1,15}.

This method is mainly employed to reduce the limitations of protein-based drugs which have a short half-life and are immunogenic for example, PEG-bovine adenosine deaminase (ADAGEN)\textsuperscript{1,2,5,9}. One of the main limitations is in the use of high molecular weight PEG since it accumulates in the liver\textsuperscript{1}.

One of the oldest PEGylation methods uses cyanuric chloride to be linked to PEG firstly, followed by the second chloride of the PEG dichlorotriazine derivative linking to amines from a protein or drug at room temperature and pH 9 to form a secondary amine linkage (Scheme 1-1, left). The main problem with this type of linkages is multiple side reactions as well as the toxicity of cyanuric chloride and its derivatives\textsuperscript{1,2,24}.

Polymers like PEG can also be linked with amines using N-hydroxysuccinimide (NHS) esters by acylation\textsuperscript{1,5,15,26,27}. The carboxylic acid group of succinylated PEG reacts with NHS ester to form succinimidyl succinate PEG which readily reacts with amines at physiological pH and hence can form amide bonds with drugs or proteins (Scheme 1-1, right). The main issue with this reaction is the hydrolysis of the ester bond of succinylated PEG which can be resolved by coupling of NHS to PEG via a carbonate group\textsuperscript{1,2,19,26,28}. One of the examples of this type of linkage is the generation of controlled-release nanoparticles from PLA-PEG block copolymers with a terminal carboxylic acid group attached to PEG conjugated with primary amine terminated aptamers\textsuperscript{27}. Well-defined NHS α-functional polymers (poly(PEGMA)) for bioconjugation with biomolecules can also be synthesised using ATRP and RAFT\textsuperscript{25}. 

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Brocchini has shown the polymerisation of diamino PEG and a bis(N-hydroxy)succinimide macromonomer derived from PEG and cis-aconitic acid leads to a water soluble polymer which degrades in acidic pH\textsuperscript{29}.

\begin{align*}
R-\overset{\text{N}}{\text{N}}\overset{\text{Cl}}{\text{N}}\overset{\text{Cl}}{\text{N}} \overset{\text{H}}{\text{R'}} & \xrightarrow{R'-\text{NH}_2} R-\overset{\text{N}}{\text{N}}\overset{\text{Cl}}{\text{N}}\overset{\text{Cl}}{\text{N}} \overset{\text{H}}{\text{R'}} \\
R-\overset{\text{O}}{\text{C}}\overset{\text{O}}{\text{C}} & \overset{\text{O}}{\text{O}} \xrightarrow{R'-\text{NH}_2} R-\overset{\text{O}}{\text{C}}\overset{\text{O}}{\text{C}} \overset{\text{O}}{\text{O}} 
\end{align*}

\textbf{Scheme 1-1.} \textit{Left:} Bioconjugation showing reaction of PEG dichlorotriazine derivative with amines via alkylation \textit{Right:} Conjugation of succinimidyl succinate PEG with amines via acylation.

Polymers can also be coupled with peptides or drugs using a single step method by the use of a carbodiimide (N,N'-diisopropyl carbodiimide (DIC) or 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)) which activates the polymer in situ. Using these methods there is a high loss of bioactivity due to the reaction being non-specific and hence having no control over the final bioconjugates\textsuperscript{1, 16, 28, 30}.

To increase the selectivity of the conjugation the active site of the enzyme or recognition area of a drug or protein can be shielded using an inhibitor, substrate or ligand during the reaction for example; avidin is PEGylated in the presence of biotin-PEG conjugate which acts as a shield\textsuperscript{1, 15}. Another way to increase the selectivity is to limit the availability of the reactive groups in the protein by replacing reactive amino acids by non-reactive ones, using mutagenesis\textsuperscript{1, 30}.

Another method which can provide specific conjugation is reductive alkylation in which an aldehyde reacts with the polymer first, followed by coupling to an amine to form a Schiff base\textsuperscript{1, 2, 31, 32}. Sodium cyanoborohydride can then be used to reduce this intermediate to a stable secondary amine. The main advantage is the functionalisation of the N-terminal amines, leaving the lyside side chain amines untouched\textsuperscript{1}. Instead of using cyanoborohydride in acidic pH, an iridium-catalysed reaction can be carried out at pH 7.4 with a high yield\textsuperscript{16, 25, 32}.

\textbf{ii) Cysteine conjugation.} Cysteine containing compounds can very well be targeted for forming bioconjugates with polymers since the free cysteines are usually low in number and hence it leads to a more site specific binding and does not result in a loss of biological activity. The main cysteine targeting reactions are thioether and disulphide forming\textsuperscript{1, 16}.
Thioether bioconjugates. Michael type addition involves a cysteine reactive reagent (vinyl sulphone) to react with thiols to form thioethers (Scheme 1-2). This is a very slow and selective reaction and even the presence of other nucleophiles like amines does not affect it. It has been used to link PEG derivatives to RNase and also to immobilise RGD proteins on surfaces\textsuperscript{1, 7, 15, 16, 33}.

Chain transfer free radical polymerisation is another method which is used together with cysteine conjugation for example; polymerisation of N-isopropylacrylamide (NIPAAm) which induces thermo-responsiveness due to its lower critical solution temperature (Scheme 1-2) followed by modification of the terminal hydroxyl group with divinyl sulphone leads to the production of a thiol-reactive polymer which can conjugate with cysteines. These stimuli-responsive materials are referred to as smart materials and can be used for diagnostic assays as in this reaction the polymer chains can be linked close to the active site of the proteins without hampering its activity\textsuperscript{1, 13, 24, 25, 31, 34-38}.

\[ RSO_{2} \rightarrow RSO_{2}SR' \]

Scheme 1-2. Bioconjugation based on reaction of vinyl sulphone with thiols to form thioethers, for example; using poly(N-isopropylacrylamide).

Instead of vinyl sulphone, maleimide is a better suited and more commonly used Michael acceptor as it reacts faster with thiols as well as is capable of reacting in slightly acidic conditions (Scheme 1-3, top). The main drawback is its products (imido groups) have low stability in water and undergo spontaneous hydrolysis, which results in heterogeneity\textsuperscript{1, 2, 15, 19, 27, 28, 33, 39}. One of the reactions using this chemistry involves reacting first an amine-terminated PEG with maleic anhydride which forms an intermediate, followed by dehydration and ring-closure by acetic anhydride and sodium acetate (Scheme 1-3, bottom). This PEG-maleimide can then react with thiols to form thioethers\textsuperscript{1, 5, 39}.
Disulphide bioconjugates. A disulphide bond is a covalent linkage which is formed by the oxidation of two sulphhydryl (SH) groups of cysteines or other SH-containing material. They are usually found in secretory proteins and exoplasmic domains of membrane proteins in bacterial and eukaryotic cells\textsuperscript{24, 40}. Bioactive compounds containing cysteines can be linked to polymers by reacting the thiol group of cysteine to an activated disulphide group on a polymer leading to a new disulphide bond. The main advantageous points of this reaction are that it can occur in a broad pH range of 3 – 10 and the newly formed disulphide bond is reversible using reducing agents like dithiothreitol (DTT) and β-mercaptoethanol\textsuperscript{1, 12, 15, 24, 28, 40, 41}. The disulphide linkage is an interesting delivery tool because of the presence of a high redox potential difference between the oxidizing extracellular space and the reducing intracellular space\textsuperscript{40}. Various activated disulphides like \textit{o}-pyridyl disulphide, \textit{p}-pyridyl disulphide, alkoxy carbonyl and \textit{o}-nitrophenyl can be used for this reaction. The most common and effective one is \textit{o}-pyridyl disulphide since it does not take part in the reaction and gets sidelined as a nonreactive compound (Scheme 1-4). Polymers like PEG can be functionalised with \textit{o}-pyridyl disulphide either by using an NHS-activated \textit{o}-pyridyl disulphide or by first activating the terminal hydroxyl groups of the polymer by \textit{p}-nitrophenyl chloroformate, followed by linking with 2-(2-pyridyldithio)ethylamine\textsuperscript{1, 5, 12, 40}. One of the first disulphide link containing drugs is Mylotarg developed by Celltech Group and American Home Products and is anti-CD33 antibody-S-S-calicheamicin, for treatment of acute myeloid leukemia\textsuperscript{40}.
Scheme 1-4. Bioconjugation of bioactive compounds to polymers by reaction of \(\sigma\)-pyridyl disulphide modified polymer with thiol containing bioactive compound to form a hetero-disulphide bond.

Instead of using the above modifications, pyridyl disulphide-functionalised initiators can be used during atom transfer radical polymerisation (ATRP) to form \(\sigma\)-pyridyl disulphide-functionalised polymers\(^1\). Disulphide-crosslinked hyaluronic acid nanogels have been developed by Park and co-workers physically encapsulating green fluorescence protein siRNA for targeting tumours\(^20,42\). Disulphide linking chemistry has also been used for the preparation of DNA-protein conjugates\(^17\).

**Bridged disulphide bioconjugates.** Proteins (leptin, interferon \(\alpha\)-2) which have cysteines in disulphide bridges on their surfaces can be conjugated with polymers like PEG by selectively reducing the surface accessible disulphide bridges using mild reducing conditions without denaturants. The two thiols which are liberated are treated with a bis-alkylating PEGylation agent which leads to the addition of the first thiol and elimination of a sulphinic acid, followed by the addition of the second thiol due to the double bond (Scheme 1-5)\(^1,41,43,44\). This addition-elimination-addition reaction ensures that the disulphide is bridged in the end linking it to the polymer and hence stabilizing the peptide\(^1,41\).
Scheme 1-5. Formation of three-carbon bridge between polymers like PEG and proteins containing disulphide surface bonds by using addition-elimination-addition reaction.

B) Grafting to using unnatural functionalities (Click reactions)

Click chemistry is defined by Sharpless et al. as a chemistry inspired by nature tailored to generate substances reliably and quickly by joining small units together. The reactions in click chemistry must (or would be desirable):

- be modular
- be wide in scope
- give high chemical yields
- generate only inoffensive byproducts
- be stereospecific
- physiologically stable
- exhibit a large thermodynamic driving force to favour a reaction with a single reaction product
- high atom economy
- have simple reaction conditions
- use readily available starting materials and reagents
- use no solvent or a solvent that is benign or easily removed (preferably water)
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- provide simple product isolation by non-chromatographic methods (crystallisation or distillation)

These are the requirements for a reaction to be classified as a click reaction. The following are few examples of click reactions:

i) **Copper-catalyzed azide-alkyne Huisgen 1,3-dipolar cycloaddition (CuAAC).** Azides and alkynes usually react by 1,3-dipolar cycloaddition at high temperatures (Scheme 1-6, top). However scientists like Huisgen have demonstrated that this reaction can be performed at room temperature and accelerated by using a copper catalyst (Scheme 1-6, bottom). It is commonly used because of its high yield, mild reaction conditions and ease of introduction of the groups to polymers and bioactive molecules. It converts organic azides and terminal alkynes into the corresponding 1,4-disubstituted 1,2,3-triazoles, whereas the uncatalyzed reaction which requires much higher temperatures leads to the formation of mixtures of 1,4- and 1,5-triazole regioisomers. One of the good examples of the use of triazoles is in the antibiotics field where they have been used to improve the pharmacokinetic properties of drugs, for example; cephalosporins endowed with good oral availability were obtained by conjugation of triazoles to cephalosporin core.

\[
\begin{align*}
R^1\text{N}^+\text{N}_2^- + \equiv R^\prime & \xrightarrow{\text{Heat}} R^1\text{N} \equiv \text{N}^+\text{N}_2^- + R^\prime \text{N}_2^- \equiv \text{N}^+ \\
& \text{1,4-triazole} \\
& \text{1,5-triazole} \\
R^1\text{N}^+\text{N}_2^- + \equiv R^\prime & \xrightarrow{\text{Cu (1) species at room temperature}} R^1\text{N} \equiv \text{N}^+\text{N}_2^- + R^\prime \text{N}_2^- \equiv \text{N}^+ \\
& \text{1,2,3-triazole}
\end{align*}
\]

**Scheme 1-6.** Top: Azide-alkyne reaction at high temperature produces 1,4- and 1,5-triazole Bottom: Azide-alkyne reaction using copper catalyst at room temperature produces 1,2,3-triazole

Alkyne groups can be introduced into polymers like PEG by coupling propargylamine with a NHS activated PEG. Bioactive molecules like superoxide dismutase-1 (SOD) can
be modified with azide by linking with $p$-azidophenylalanine. The CuAAC reaction is then carried out in a phosphate buffer at pH 8 to form PEGylated SOD$^2$. Clickable polymers can also be synthesised by using ATRP, ring-opening polymerisation (ROP) or reversible addition-fragmentation chain transfer polymerisation (RAFT) by using alkyne or azido functional initiators$^{1, 21, 22, 31, 50, 52, 53}$. Tirrell et al. have used this technique for selectively labelling expressed recombinant proteins or membrane proteins in living E. coli bacteria in their native environment. Others, like Rutjes have conjugated alkyne-functionalised bovine serum albumin to azide-functionalised polystyrene$^{16, 21, 46, 54}$. Fokin and Finn have showed how this reaction can be carried out in water and the conjugation of fluorophores to virus particles$^{19, 21, 52}$. The main disadvantages of this reaction are the toxicity of copper to cells as well as the denaturation of proteins induced by copper$^{1, 15, 19, 34, 54}$.

ii) **Hydrazone and oxime ligation.** Ketones and aldehydes can be used for conjugating peptides or drugs with polymers as they react with hydrazides and hydroxylamines respectively to form hydrazones and oximes (Scheme 1-7). These reactions are well suited for bioconjugation since the groups need no protection while conjugation since the reaction can be carried out successfully in slightly acidic conditions to prevent the side reactions (formation of Schiff base by reaction between amino groups and ketones or aldehydes)$^{1, 15, 32, 45, 46, 51}$.

![Scheme 1-7](image)

**Scheme 1-7.** Left: Bioconjugation using Ketone-hydrazide reaction forming hydrazone bond Right: Ketone-hydroxylamine reaction forming oxime bond.

Polymers like PEG can be modified with hydrazides and then conjugated with bioactive molecules containing ketones or aldehydes by a hydrazone bond under acidic conditions. This link is thermodynamically unstable and reversible and needs to be reduced by sodium cyanoborohydride to form a stable alkyl hydrazide. Similarly, a ketone or aldehyde-functionalised bioactive molecule can be linked to a hydroxylamine-bearing polymer by an oxime bond which is more stable than the hydrazone bond$^{1, 15, 19}$. 

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The half-life of alkylhydrazones and acylhydrazones is only about an hour at physiological pH while the half-life of oximes is nearly a month. Since many proteins are unstable at low pH, the reaction can be performed at neutral pH by the addition of p-methoxyaniline which acts as a nucleophilic catalyst and accelerates the rate of reaction.

Controlled hydroxylamine radical initiators can be used for synthesis of polymers with hydroxylamine groups using ATRP, for example; tert-butyloxycarbonyl (Boc) protected hydroxylamine initiators can be used for synthesis of HEMA and poly(ethylene glycol) methacrylate (PEGMA).

iii) **Copper-Free cycloaddition reaction with azides.** Literature has shown that activated alkynes can undergo cycloaddition reactions with azides in the absence of a metal catalyst. Alkynes with at least one electron-withdrawing group can react with 5-azidovalerate at room temperature in water giving a yield of 67 – 94% (Scheme 1-8).

![Scheme 1-8. Click reaction between azides and electron-deficient alkynes (R' = H, CH₃, or COOEt, R'' = Me or Et, and N₃-X = 5-azidovalerate or 5'-azido-DNA).](image)

This click reaction has also been performed to couple an azido-DNA molecule to prove that functional groups can be introduced to DNA under physiological conditions.

iv) **Thiol based click reactions.** The radical based addition of thiols to double bonds is a well known method used for polymerisation, curing reactions and for the modification of polymers. One of the most simple thio-click reactions was shown by Schlaad et al. for a post-polymerisation modification of poly[2-(3-butenyl)-2-oxazoline] (Scheme 1-9). This reaction was performed under an inert atmosphere by exposure to UV light for 24 hours, but can also proceed under irradiation with direct sunlight. Various other thiols like fluorinated thiols and acetylated glucose thiols have also been used in this reaction.
Another thiol based click reaction was performed by Hawker et al. for the synthesis of fourth generation dendrimers by using thiol-ene chemistry. The reaction between an alkene and a thiol was performed without a solvent under ambient conditions by irradiation with a UV lamp for 30 minutes (Scheme 1-10). Trace amount of photoinitiator was added to increase the concentration of radicals, and purification was carried out by precipitation in diethyl ether. 

v) **Diels-Alder reaction.** Diels-Alder reactions involve the simultaneous formation and destruction of carbon-carbon bonds. They require very low energy and can carried out even below room temperature. One of the most common types of Diels-Alder bioorthogonal reactions was reported by Fox et al. for the reaction between tetrazines and cyclooctynes (Scheme 1-11). This is based on the inverse electron demand Diels-Alder reaction, and the subsequent retro-[4+2] cycloaddition produces nitrogen as the
only byproduct. The reaction takes 40 minutes at 25 °C to give a high yield and can be performed in water\textsuperscript{34}.

\begin{center}
\begin{align*}
\text{H} & \quad \text{H} \\
& \quad + \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
& \quad \text{N}_2 \\
& \quad 25 \, \text{°C}, \, 40 \, \text{min}
\end{align*}
\end{center}

\textbf{Scheme 1-11.} Retro-Diels-Alder reaction of trans-cyclooctene and tetrazine.

C) Grafting to using non-covalent interactions

Bioconjugates can also be formed without covalent coupling, by using coordination strategy between polymers and bioactive compounds by the use of cofactors. Cofactors, which are present in the active site of proteins and responsible for protein activity can be removed and attached to polymers. These polymer-functionalised cofactors when mixed with proteins lead to the formation of bioconjugates as the cofactor is reconstituted in the protein\textsuperscript{1, 17, 31, 37}.

One of the examples of this type of bioconjugation is the biotin-(strept)avidin system. Biotin is a cofactor which has a very high affinity for avidin and streptavidin. Biotin contains a carboxylic acid group which can easily link with various polymers and hence bioconjugates can be synthesised using avidin or streptavidin. This linkage is one of the strongest and hence it is used for constructing materials such as DNA or protein microarrays. Biotin can also be incorporated into the initiators for ATRP and RAFT thereby leading to biotin-functionalised polymers\textsuperscript{1, 17, 28, 31, 37}.

In drug delivery, PEG-based heparin-functionalised star-polymers have been cross-linked with dimeric protein vascular endothelial growth factors (VEGF) to form hydrogels. VEGF can bind to heparin and also acts as a crosslinker for the gel formation. When particles covered with VEGF receptors bind to VEGF proteins, erosion of the hydrogel is triggered and this could release the incorporated drugs\textsuperscript{1}. 
D) Grafting from using polymer macroinitiators

There are lots of techniques for bioconjugation of preformed polymers to bioactive molecules; however a polymer can also be grown from these bioactive molecules which can act as initiators. The main advantages of using this strategy are the need for a small amount of initiator, lower steric hindrance during functionalisation and simple purification due to removal of only the monomer\(^1,25,31,37,51,53\).

One of the main strategies for growing a polymer bioconjugate is using a primary amine-functionalised initiator, for example; primary amine-functionalised initiators are used for ring opening polymerisation (ROP) of amino acid \(N\)-carboxyanhydrides (NCAs) forming poly(amino acids) (Scheme 1-12, top). This reaction is not controllable due to chain-breaking and termination reactions in normal conditions, but can be controlled by using high vacuum or by replacing the primary amine initiator by its hydrochloride salt\(^1,25,54,55\).

An alternative reaction to the above uses a zero-valent nickel complex as initiator to carry out NCA polymerisation (Scheme 1-12, bottom). Through an oxidative addition-reductive elimination mechanism, nickel allows the addition of monomers to the active polymer chain-end and also prevents any side termination reactions\(^1,37,54,55\).

Other more complex macroinitiators containing arms of amines can also be used for the growing polymers, for example; star-shaped PEG with four terminal amine groups has

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Scheme 1-12. *Top:* ROC of NCAs using a primary amine initiator *Bottom:* ROC of NCAs using a nickel catalyst [2,2'-bipyridine – Nickel – 1,5-cyclooctadiene].
been used for ROP of γ–benzyl-L-glutamate NCA to yield poly(γ–benzyl-L-glutamate)\textsuperscript{1, 25, 37, 54}.

Börner has investigated the interactions of ATRP systems with oligopeptide macroinitiators using homogenous solution-phase ATRP for the synthesis of poly(n-butyl acrylate) as well as used RAFT polymerisation for synthesis of bioconjugates\textsuperscript{53, 54}.

E) Grafting through

Bioconjugates can also be prepared in comb-like structures with a polymer backbone and bioactive side chains by linking the bioactive molecules to the monomers and then carrying out polymerisation. The main advantage of this strategy is quantitative functionalisation, while the disadvantage is that there might be compatibility issues between the bioactive molecule and the polymerisation technique\textsuperscript{1, 25, 37}.

An example of this strategy is the ATRP polymerisation of a methacrylate coupled to a pentapeptide (Val-Pro-Gly-Val-Gly) derived from the structural protein tropoelastin using the initiator Ebib (ethyl-2-bromo-2-methyl-propionate) (Scheme 1-13). This polymerisation is controllable and yields polymers with low polydispersity index\textsuperscript{1, 37}.

\chem{\begin{array}{c}
\text{O} \\
\text{Br}
\end{array}}
\text{O} \\
\text{O} \\
\text{O} \\
\text{N}
\text{Peptide}
\text{Cu catalyst}
\text{O} \\
\text{O} \\
\text{Peptide}
\text{Br}
\text{O}

\text{O}
\text{O}
\text{Peptide}
\text{Peptide}

Scheme 1-13. ATRP polymerisation of a methacrylate coupled to a pentapeptide using Ebib initiator.

F) pH Sensitive linkages

In the body, tumours and inflammatory tissues have a pH more acidic than blood and normal tissue. The endocytic pathway of cells has a range of pH, whereby it starts from 7.4, and then lowers down to 5.5 – 6 in the endosomes and finally a pH of 4.5 – 5 in the
lysosomes. This drop in pH can be used as a favourable aspect for drug delivery systems which are pH responsive2, 11, 13, 56-59.

**Acetal links.** Acetal linkages are acid-sensitive linkages for linking polymers with hydroxyl groups of bioactive compounds and are reasonable for drug delivery since their hydrolysis rate follows first order kinetics relative to hydronium ion, in other words they hydrolyse 10 times faster with each unit of decrease in pH56, 60-62. They can be formed using various different types of hydroxyl groups such as primary, secondary, tertiary, syn-1,2- and -1,3-diols. Also depending on the structure of the acetal, the hydrolysis rate can be controlled56, 61-64. Ketones react with hydroxyl groups from alcohols to form an intermediate (hemiacetal) which becomes protonated and further reacts with alcohols to form acetals (Scheme 1-14)60, 65. This reaction also leads to the production of water which needs to be removed to prevent hydrolysis of the acetal. These acetals hydrolyse very quickly with a half-life of 90 seconds at pH 5 and 6 hours at pH 7.4 at 25 °C and are mostly used for rapid targeting to tumours where they can release the bioactive compound even in slightly acidic extracellular tumour pH56.

One of the examples of this ketone-alcohol reaction to form acetals is shown by Fréchet where he shows the bioconjugation of activated poly(ethylene oxide) (PEO) to 5-fluoro-2'-deoxyuridine (anticancer drug) by forming an acetal link56.

![Scheme 1-14. Synthesis of acetals from ketones by using alcohols.](image)

Aldehydes react with alcohols as well to form acetals, and these acetals are much more stable and slow hydrolysing than the acetals formed from ketones because of the absence of the second electron-donating methyl group which stabilises the carbocation intermediate during hydrolysis64. The acetal obtained from acetaldehyde and primary alcohol has a half-life of 100 hours at pH 5 and 3 years at pH 7.4, and hence these acetals can be used for applications where they need to be stable at physiological pH for long periods56. An example for drug delivery by using this strategy is by synthesising...
ATRP based poly(ethylene oxide)-block-poly(glycerol monoacrylate) (PEO-b-PGA) and then coupling with drugs like 1-pyrenecarboxaldehyde using acetal linkage\textsuperscript{66}. Acetal linkages have also been used for formation of responsive gels, for example; PEG has been grafted on a polyacetal backbone to form a temperature/pH-sensitive gel which disintegrates at acidic pH\textsuperscript{63}. For drug delivery, acetalated-dextran which is hydrophobic has been used for encapsulating hydrophobic and hydrophilic drugs since it is acid-sensitive and biocompatible\textsuperscript{67}. Fréchet has synthesised acid-sensitive micelles made from PEO-dendrimer hybrids which incorporate hydrophobic groups linked to the periphery of the block by acetal links; such that upon hydrolysis, the hydrophobic groups are lost, resulting in destabilization of the micelle and hence release of drug\textsuperscript{57,59}. Another very interesting strategy has been used by Duncan, where oestrogen diethylstilboestrol (DES - anticancer drug) has been incorporated into the mainchain of water-soluble polyacetals synthesised using co-monomers of PEG resulting in a drug release system by degradation of the polymer backbone in the lysosomes of the tumour\textsuperscript{68}. Duncan and Brocchini have also shown the preparation of polyacetyl-doxorubicin conjugates for tumour targeting by synthesising amino-pendent polyacetals and coupling with doxorubicin by using EDC\textsuperscript{62}. Brocchini has also prepared polyacetals by the reaction of a diol (e.g., PEG) with divinyl ether (e.g., tri(ethylene glycol) divinyl ether) using an acid catalyst\textsuperscript{69}.

**Boronic acid – Diol links.** Boron containing molecules have been found to have biochemical functions in plants, algae and microorganisms but are not known for any significant role in mammalian cells\textsuperscript{70}. Boronic acids bind with diol containing compounds through a reversible reaction leading to boronate ester formation (Scheme 1-15). Various methods have been used in the past for the detection of boronate ester formation such as by appending a fluorophore to the boronic acid, nuclear magnetic resonance, circular dichroism, and pH titration. The problem associated with appending a fluorophore directly to the boronic acid is that it could greatly affect the binding affinity of the artificial receptor for the target sugar in a negative fashion while the issue with NMR is that, it usually lacks the desired sensitivity to evaluate the binding strengths\textsuperscript{71}. The stability of this link is pH dependant and it affects the binding affinities of boronic acids towards various diols. This property of pH responsiveness enables boronic acids to be used for creating sensors for saccharides which contain cis-diol moieties and for delivery of bioactive substances when linked with polymers\textsuperscript{71-77}. 


Boronic-diol conjugation can be regarded as a click reaction since it meets most of the criteria (see Section 1.1.2 Part B) for it, for example: the joining of small units quickly and reliably, wide in scope, give high yield, generate only inoffensive byproducts, physiologically stable – as these links are stable at pH 7, simple reaction conditions – as it can be performed at room temperature, use readily available starting materials and regents, use a solvent like water, provide simple product isolation – as they can be separated easily especially when one is linked with a polymer. Since it meets a lot of the desirable criteria, we can claim the boronic-diol conjugation to be a click reaction.

They have also been used in new technologies like BASE (boronate affinity in saccharide electrophoresis) which relies on reversible diol binding to modulate saccharide mobility. Hydrogels made from this technology are common as well since they possess self-healing property i.e. under high shear stress the boronate-diol network breaks up (shear thinning) but reheals quickly when it comes to rest. The optimal pH of binding where the equilibrium binding constant is the highest is the average of the pKₐ’s of the boronic acid and diol. As shown by Wang, the Kₑq (equilibrium binding constant) values for α-glucose, α-fructose and catechol rise from acidic pH till pH 8.5. The Kₑq values for α-fructose at pH 5.8, 7.4, and 8.5 are 4.6, 160, and 560 M⁻¹. Wang also shows that, boronic acids like phenylboronic acid have an increasing affinity for diols like alizarin red-S (ARS) when increasing pH from 4-7 and that the maximum binding obtained is at pH 7 (Kₑq = 1100). On increasing the pH from 7-10 a drop in the binding affinity is found probably due to change in ionization states when it changes from trigonal to tetrahedral form. It is well known in literature that boronate-ester complexes have decreased stability under acidic conditions which results in a high concentration of the quenched free form of ARS (Kₑq = 190 at pH 4.6) at low pH (<5). Therefore, ARS displays a dramatic change in fluorescence intensity and colour in response to the binding of a boronic acid and is used as a general reporter for studying carbohydrate-boronic acid interactions, both quantitatively and qualitatively.

This property of boronic acids to bind strongly only at physiological or basic pH, and showing reversibility at acidic pH could be used efficiently to delivery drugs when coupled with a degradable polymer. The idea would be that a boronic acid could be linked by a non-reversible bond to a degradable, high molecular weight biopolymer, followed by reversibly conjugating with a diol based drug at physiological pH, the conjugate would then be purified to remove any excess unbound drug, and will then be
injected into the bloodstream where the conjugate would be stable due to the physiological pH and would circulate through the body, until it arrives at the site of cancer/tumour/inflammatory tissue, where the conjugate would be endocyted by the cell (due to cell surface receptors) and the drug would be released in the late endosome/lysosome of the cell where there is an acidic pH (due to acidic pH the boronic acid-diol reaction reverses, releasing the drug) whereas the polymeric carrier would be degraded by enzymes.

An example of this is provided by Gan et al. who have prepared a magnetic and reversible pH-responsive mesoporous silica nanoparticles-based (MSNs) nanogated ensemble by anchoring superparamagnetic Fe₃O₄ nanoparticles on the pore outlet of MSNs via a reversible boronate ester linker. They have studied the in vitro release of an entrapped model dexamethasone at different pH values which indicated that at acidic pH (<5) the hydrolysis of the boroester bond took place and it resulted in a rapid release of the drug. In vivo studies were also carried out to evaluate the cytotoxicity of the cells with the nanoparticles, but no release profiles were shown³².

The binding of boronic acids to diols has been studied in detail in Chapter 2. Polymers can be functionalised with boronic acids and can be used for drug delivery by bioconjugation to bioactive compounds like drugs or peptides containing diols. This is studied in detail in Chapter 3.

\[
\begin{align*}
\text{Scheme 1-15. Reversible linkage of phenylboronic acid to a diol-functionilised moiety showing the trigonal and tetrahedral forms.}
\end{align*}
\]
1.2 Hyaluronic acid (HA) and its functional derivatives

Hyaluronic acid (HA) also known as hyaluronan or hyaluronate is a naturally occurring glycosaminoglycan found in the extracellular matrix in the body\textsuperscript{83, 84}. It is known as hyaluronic acid due to its location in the body and its chemical nature. It was first discovered in the vitreous humour of the eye and therefore the name hyaloid which means vitreous and it contains uronic acid, therefore the name hyaluronic acid. HA exists in the body as a polyanion and not in the acid form and is therefore usually known by the name of hyaluronan\textsuperscript{85, 86}.

1.2.1 Structure of HA

HA is a high molecular weight polysaccharide with disaccharidic repeating units composed of D-glucuronic acid and N-acetyl-D-glucosamine monomers (Scheme 1-16). These units are linked together by a 1-4 $\beta$ glycosidic bond, while the disaccharides are connected by 1-3 $\beta$ bonds in the polymer chain\textsuperscript{87, 88}.

![Repeating Unit](image)

Scheme 1-16. Structure of hyaluronic acid

The main difference of HA from other glycosaminoglycans is the absence of sulphate groups\textsuperscript{89}. HA usually has a molecular weight of a few million g/mol and a broad molecular weight dispersity. The end to end distance in a linear conformation in HA is approximately 2-25\mu m\textsuperscript{83}.

The carbon-hydrogen bonds in the saccharidic repeating units are all in axial configuration; therefore they form a hydrophobic face, while the equatorial, polar
residues form a hydrophilic face, providing therefore a two-face, two-polarity ribbon-like structure. Although the HA molecule looks like a straight rigid rod, however when magnified it is a coiled structure. Since it is very rigid, the coils take up more number of units per turn and therefore the structure has a lot of empty spaces, which are filled up with water. The structure has alternating apolar faces which might interact with the apolar faces on another molecule but all the apolar faces cannot couple up because there will be a drastic loss in the entropy.

The molecule has a coil-like structure when in solution. This coil-like structure is produced by the occurrence of hydrophobic and hydrophilic groups in the solution. This structure allows smaller molecules to pass through, however large molecules like proteins will have restricted access. Usually in tissues high molecular weight HA at high concentration can form entangled molecular networks. Such networks can be formed if the hydrophobic face on a section of HA interacts reversibly with the hydrophobic face on another molecule of HA or another region of the same molecule. These networks can exhibit elastic properties by distributing the load and shear forces within the network and thereby resisting rapid and short duration fluid flow through the network. They can exhibit viscous properties by separating and aligning their molecules and thereby allowing slow fluid flow of longer duration.

1.2.2 Occurrence, Functions and Commercial Products of HA

Hyaluronan was first found to be present in the vitreous humour of the eye. Since then it has been located in many places such as the skin and in some joints (Figure 1-1). It is present in reasonable quantities in aortic smooth muscle cells and chondrocytes and also in the nuclei and cytoplasm of cells of some of the tissues.

*Figure 1-1. Occurrence of hyaluronic acid in Joints, vitreous humour of the eye and skin (Taken from)*
HA is present in tissues such as vocal folds, synovial fluid, umbilical cord, dermis, subcutaneous tissue and cartilage where it performs a number of functions\textsuperscript{89, 97, 98}. In tissues where HA is present, it performs a role of contributing towards tissue viscosity, tissue flow, tissue osmosis, shock absorption, wound healing and space filling\textsuperscript{97}. HA is also metabolically active and cells focus attention on the processes of its synthesis and catabolism\textsuperscript{99}. So basically this leads us to the conclusion that HA performs 2 main functions: one where there is a high concentration of HA it performs various functions by interactions and second where there is a low concentration of HA it acts as a structural element\textsuperscript{85}. The companies which produce HA commercially and their products are detailed in the pie chart below, with the major producer being the Japanese company Seikagaku (Figure 1-2).

![Figure 1-2](image_url)
1.2.3 Receptors of HA

HA plays an important role in cell proliferation and migration. Cells interact with hyaluronan through cell surface receptor with an intermediate known as hyaluronan binding proteins. The hyaluronan binding proteins are known as hyaladherins. The two most common known receptors which have been characterised are CD44 and RHAMM (receptor for HA mediated motility). Some of the other known receptors are Layilin, ICAM-1, IVD4 and LEC.

CD44. CD44 is a proteoglycan and one of the most common receptors of HA which has been characterised. It belongs to a family of leukocyte antigens which are known as clusters of differentiation (CD). CD44 is expressed by almost all cells of neurectodermal origin where majority of primary cells, except epithelial cells express the standard form of CD44. Malignant derivatives of many cells also express variant isoforms of CD44.

Many primary cells like leukocytes do not bind to HA until they are activated, whereas fibroblasts, smooth muscle cells and most primary tumour cells have CD44 which is always activated.

Certain experiments have shown that CD44 can bind to HA in vitro and that it has a higher affinity to larger molecules of HA. CD44 is also responsible for growth factor and chemokine binding and presentation.

CD44 has four functional domains – namely the distal, proximal, transmembrane and cytoplasmic domains (Figure 1-3).

Figure 1-3. The Functional Domains of CD44 Receptor (Taken from)
Chapter 1

The distal domain is responsible for binding hyaluronan while the proximal domain is responsible for the alternate splicing of mRNA. The CD44 gene contains 19 exons out of which 10 are variably spliced. Several isoforms arise due to this alternate splicing which range in size from 80 to 250kDa\textsuperscript{103, 104, 107, 108}. CD44 undergoes modifications like phosphorylation, glycosylation and attachment of glycosaminoglycan chains. These modifications are known as post-translational modifications and they contribute to binding of hyaluronan\textsuperscript{103, 104}. There are three states of CD44 activation: active CD44 which is least glycosylated, inactive CD44 (does not bind to HA even in the presence of inducing factors) which is most glycosylated and inducible CD44 (activated by inducing factors like cytokines) which is partially glycosylated\textsuperscript{106}. Some studies have suggested that the presence of basic amino acids in the distal domain is essential for the binding of CD44 to hyaluronan. Also arginine 41 was discovered as a critical component required for the binding\textsuperscript{104}.

CD44 has a variety of functions ranging from cell-cell aggregation, retention of pericellular matrix, matrix-cell and cell-matrix signalling, receptor mediated degradation of hyaluronan, and cell migration\textsuperscript{104, 107}. Apart from its function as a HA receptor, it also acts in lymphocyte activation and lymphogenesis\textsuperscript{101, 109}.

**RHAMM.** RHAMM belongs to the family of itinerant hyaladherins. RHAMM is another common receptor of HA which occurs on the cell surface, in the cytoskeleton, in the mitochondria and in the nucleus of cells. Both the forms of RHAMM, the surface RHAMM and intracellular RHAMM are required for cell motility and cell proliferation in both normal as well as tumour cells. RHAMM has also been found on ras-transformed cells which increase cell motility by the use of antibodies\textsuperscript{101, 110-113}. There are 3 basic forms of RHAMM – Long RHAMM, alternatively spliced RHAMM and short RHAMM. They differ in their sizes and their reactions with antibodies. The structure of RHAMM is divided into various domains namely D1, D2, D3, D4 and D5 (Figure 1-4). These Domains are required for cell motility and they contribute to various interactions including activation of kinases and binding with hyaluronan\textsuperscript{110, 112}. Intracellular RHAMM proteins may connect actin and microtubule cytoskeleton due to their ability to form coils as well as it limited homology with proteins that link microtubules and actin filaments\textsuperscript{113}.
RHAMM, like CD44 also contains sites for post-translational modifications like N-glycosylation sites and phosphorylation sites\textsuperscript{110}. Although it is known that CD44 and RHAMM can participate individually in proliferative and migratory functions, they have overlapping functions in some situations and hence their individual roles cannot be clearly specified\textsuperscript{112}.

Studies on animal models for metastases have shown the dependence of hyaluronan on it; especially in the case of tumour cells which have been treated with soluble RHAMM are unable to form long metastases\textsuperscript{112}. Using biotinylated HA, RHAMM has been shown to be the main HA binding protein in cell lysates of ras transformed fibroblasts and smooth muscle cells responding to wound injury\textsuperscript{114}.

**Layilin.** Layilin is a widely expressed integral membrane hyaluronan receptor which binds to HA through its extracellular domain and is present in various cell types and tissues. It is capable of mediating cell adhesion. Though the exact function of layilin remains unclear, it is hypothesised that it might mediate early interactions between spreading cells and the ECM. Apart from binding to HA it can also bind to cytoskeleton membrane like proteins through its cytoplasmic domain\textsuperscript{115,116}.

**ICAM-1.** The intercellular adhesion molecule is expressed by various cell types like leukocytes and endothelial cells. It is easily inducible by cytokines like interferons and inhibited by glucocorticoids. Hyaluronan is one of the main ligands for ICAM-1 which
plays a role in inflammatory processes and in T-cell mediated host defence system\textsuperscript{117-120}.

### 1.2.4 Synthesis of HA

Hyaluronan synthesis is a high energy consuming process for cells as it requires a reasonable number of NAD cofactors, acetylCoA and monosaccharide groups. HA is the only glycosaminoglycan which is synthesised in the plasma membrane\textsuperscript{121}. The HA synthases or HASs are glycosyltransferases, present in the plasma membrane which regulate the polymerisation of HA and its transfer into the extracellular matrix. There are three types of HASs: HAS1, HAS2 and HAS3\textsuperscript{121}. The three hyaluronan synthases are synthesised during different stages of development and their functions/expressions are highly dependent on the tissues/cells\textsuperscript{122, 123}. HAS1 is responsible for the formation of both HA and chitin. Chitin is important for the formation of two distinct glycosidic linkages to form the repeating unit of the disaccharide\textsuperscript{121}. HAS1 is independent and can synthesise HA without the use of any proteins\textsuperscript{122}. HAS1 has a lower rate of production of HA as compared to the other HASs. This means that the there is lower concentration of HA in the matrix around the cells. This is also proved due to the fact that the half life of HAS1 is lower than the other HASs\textsuperscript{123}. The growth of the HA chain takes place by addition of alternating monosaccharides to its reducing end. In this process the growing HA chain remains covalently attached to a terminal uridine 5’-diphosphate (UDP) and the new monosaccharide is attached to a second UDP on the reducing end, which then displaces the terminal UDP. As the HA chain grows, the end opposite to where the new monosaccharides are added is pushed through a pore outside the plasma membrane\textsuperscript{99, 122}. With this process the HA does not have limited growth and can proceed to form long HA chains\textsuperscript{99}. However increased synthesis of HA is usually linked to cancerous tumours\textsuperscript{123}. HAS2 is responsible for the synthesis of hyaluronan required for the development of extracellular proteoglycan complexes in the cartilage like the valves of the heart\textsuperscript{102, 122}. HAS3 is responsible for the production of hyaluronan which is of a lower molecular weight (1×10\textsuperscript{5} to 1×10\textsuperscript{6} Da) than the hyaluronan produced by HAS2 (2×10\textsuperscript{6} Da) and HAS1 (2×10\textsuperscript{5} to ~2×10\textsuperscript{6} Da)\textsuperscript{123}. This low molecular weight hyaluronan can be used more effectively for cell signalling than higher molecular weight hyaluronan\textsuperscript{102, 122}.  

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117-120. 

121. 

122. 

123. 

99. 

102. 

122. 

123.
1.2.5 Endocytosis, Degradation and *in vitro* and *in vivo* studies of HA and its conjugates

Endocytosis is the widespread cellular function that regulates the quantal uptake of exogenous molecules from the cell’s environment via plasma membrane-derived vesicles and vacuoles since most substances important to cells are large polar molecules that cannot pass the hydrophobic plasma or cell membrane. There are four main categories of endocytosis pathways:

**Clathrin-mediated endocytosis.** It is mediated by vesicles which are approximately 100nm in diameter and have a characteristic crystalline coating made up of a complex of proteins which are mainly associated with the cytosolic protein clathrin. These vesicles are found in almost all cells and form domains of the plasma membrane termed clathrin-coated pits which can concentrate large extracellular molecules that have different receptors responsible for the receptor-mediated endocytosis of ligands, for example: transferrin, growth factors, antibodies, etc.

**Caveolae.** They consist of the cholesterol-binding protein caveolin with a bilayer enriched in cholesterol and glycolipids. They are flask shaped pits in the membrane approximately 50nm in diameter. They can take up almost a third of the plasma membrane area of the cells of some tissues, and are found mainly in smooth muscle, fibroblasts, adipocytes, endothelial cells and type 1 pneumocytes. They are believed to be responsible for the uptake of extracellular molecules.

**Macropinocytosis.** It usually starts from the plasma membrane and is the folding of the cell membrane to form a pocket, which then pinches off into the cell to form a vesicle (0.5 – 5 µm in diameter) filled with large volumes of extracellular fluid and molecules within it. The vesicle then travels into the cytosol and fuses with endosomes and lysosomes.

**Phagocytosis.** All particulate matter which is larger than 0.75 µm in diameter is internalised by phagocytosis. This includes small dust particles, cell debris, microorganisms and even apoptotic cells.
The principal components of endocytic pathway are: early endosomes, late endosomes, and lysosomes. Early endosomes are the first in line and receive most of the vesicles coming from the cell surface, they have a mildly acidic pH and they are the sorting organelles for separating the ligands from their receptors. Late endosomes receive internalised material which is en route to lysosomes, usually from the early endosomes in the endocytic pathway, from trans-golgi network in the biosynthetic pathway, and from phagosomes in the phagocytic pathway. They contain lysosomal membrane glycoproteins and acid hydrolases, and they have a pH of approximately 5.5. Lysosomes are the last compartment of the endocytic pathway with a pH of about 4.8 and appear as large vacuoles with a high content of lysosomal membrane proteins and active lysosomal hydrolases. It principally serves to break down cellular waste products, fats, carbohydrates, proteins and other macromolecules into simple compounds by using about 40 different kinds of hydrolytic enzymes; which are then recycled to the cytoplasm as new cell-building materials.\textsuperscript{124, 126, 127}

Endocytosis of HA usually takes place either within the tissue where it is synthesised or in the lymph nodes and the liver so as to be replaced with fresh HA.\textsuperscript{122, 129} The degradation of HA takes place by binding of an HA molecule to an HA receptor (like CD44), followed by the receptor complex being taken into the cell via endocytosis, and finally the HA molecule is degraded into its monosaccharide units by hyaluronidase in the lysosome.\textsuperscript{88, 97, 105, 130} CD44 can mediate HA endocytosis during morphogenesis of tissues such as the lung and skin, during long bone growth, and in adult tissues such as cartilage.\textsuperscript{100} However, the most efficient HA receptor responsible for internalisation and degradation is the liver endothelial cell receptor that forms a large molecular mass complex of several subunits. LYVE-1, a lymph vessel-specific receptor has also been shown to participate in the endocytosis of HA in the lymphatics.\textsuperscript{86, 88, 122}

The mechanism of HA uptake by cells appears unique and is still being researched on because clathrin-coated pits and caveolae, the most common vehicles for endocytosis, seem not to work in this case.\textsuperscript{84, 100} The exact reasoning for why some HA-CD44 interactions signal, some promote endocytic uptake, and other permit retention of HA on the cell surface has not yet been resolved.\textsuperscript{122} A few investigators have related the cellular capacity for CD44-mediated endocytosis and degradation of HA as assessed \textit{in vitro} to tumour metastatic aggressiveness.\textsuperscript{104}

The degradation of HA \textit{in vivo} is carried out by three classes of enzymes: Hyaluronidase, \(\beta\)-D-glucuronidase and \(\beta\)-N-acetyl-D-hexosaminidase. Basically,
hyaluronidase degrades the HA and the oligosaccharides produced by the degradation are degraded by the other two enzymes. The minimum degraded unit by this mechanism is the disaccharidic repeating unit\textsuperscript{88, 131}.

Hyaluronidase is an enzyme which breaks the β-N-acetyl-D-glucosamine bonds of the HA separating the polysaccharides N-acetyl glucosamine and glucuronic acid. The activity of Hyaluronidase depends on the density of carboxylate (COO\textsuperscript{−}) groups. For example, non-esterified HA and partially esterified HA can be degraded by hyaluronidase, while completely esterified HA cannot be degraded\textsuperscript{131, 132}.

The degradation of HA is reduced in the presence of chondroitin sulphate and heparin as they can bind to hyaluronidases and reduce their activity on HA\textsuperscript{131, 133}.

HA can be hydrolysed by mammalian hyaluronidases to form tetra and hexasaccharides.

There are six gene sequences related to hyaluronidase: HYAL1, HYAL2, HYAL3, HYAL4, PHYAL1 and SPAM1. Their correspondence to the Hyaluronidase is mentioned in the Table below:\textsuperscript{84}

**Table 1-1.** Gene sequences and their corresponding Hyaluronidase

<table>
<thead>
<tr>
<th>Gene</th>
<th>Corresponding Hyaluronidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYAL1</td>
<td>Hyaluronidase-1</td>
</tr>
<tr>
<td>HYAL2</td>
<td>Hyaluronidase-2</td>
</tr>
<tr>
<td>HYAL3</td>
<td>Hyaluronidase-3</td>
</tr>
<tr>
<td>HYAL4</td>
<td>Hyaluronidase-4</td>
</tr>
<tr>
<td>PHYAL1</td>
<td>Transcribed but not translated in human</td>
</tr>
<tr>
<td>SPAM1</td>
<td>PH-20</td>
</tr>
</tbody>
</table>

Hyal-1 found in lysosomes, can degrade HA to tetrasaccharide units or smaller disaccharides and can suppress tumours as it can act as a TSG (Tumour Suppressing Gene) as eradication of hyaluronidase activity is an important step in the development of some cancers\textsuperscript{84, 122}.

Hyal-2 is attached to the plasma membrane and can degrade HA to a molecular weight of about 20kDa. Hyal-2 can act either as a TSG or as an oncogene\textsuperscript{84, 122, 133}.

Hyal-3 has been found in the chondrocytes but its responsibilities/activities have not yet been identified. It is widely present and its expression is enhanced during chondrocyte differentiation\textsuperscript{84, 133}. 
Chapter 1

Hyal-4 is a peculiar hyaluronidase enzyme that can degrade HA but its activity on HA is minimal as it can attach to chondroitin and chondroitin sulphate which inhibit it\(^{84, 133, 134}\).

The gene PHYAL1 is not translated into an enzyme in humans however in other species it does translate. It is a pseudogene with multiple deletions and contains an abnormal stop codon, however not much is known about it\(^{84, 133}\).

PH-20 plays a variety of roles and has been found distributed in various regions of the body. One of its main occurrences is in the testes, however it is also found in the epididymis, the female genital tract, breast, placenta and fetal tissues. PH-20 also plays an important role in fertilization. It also expresses itself in some cancers\(^{84, 122, 133, 134}\).

Various types of tumours like epithelial, ovarian, colon, stomach and acute leukaemia, over produce HA such that elevated HA contents are prognostic for malignant progression\(^{135}\). CD44 and RHAMM are overexpressed in many types of cancer cells, demonstrating enhanced binding and internalisation of HA\(^{136, 137}\).

The delivery of doxorubicin (DOX) to several cancer cell lines (human breast, ovarian and colon) has been shown \textit{in-vitro} by N-(2-hydroxypropyl)-methacrylamide (HPMA) polymer grafted with both DOX and low molecular weight HA by using a lysosomally degradable spacer. They showed selective toxicity towards cancer cells overexpressing HA receptors whereas free doxorubicin as well as HPMA-DOX conjugate were toxic to both cancer cells and to fibroblasts\(^{138}\).

Paclitaxel (Taxol) has been linked at 2’-OH by a succinate ester spacer to low molecular weight HA since the drug is poorly soluble in aqueous media. This conjugate was targeted \textit{in vitro} to human cancer cell lines (HBL-100, SK-OV-3 and HCT-116) and only CD44 overexpressing cells internalised the conjugate after 20 minutes of incubation. The ester linkage was found to be stable in the media with cells while in the presence of esterase it rapidly released the drug, which bought forward the conclusion that the conjugate is first internalised by receptor-mediated endocytosis, followed by esterase-catalysed drug release in the lysosomal compartment. The human cancer cells were selectively killed, while the fibroblasts were unaffected\(^{139, 140}\).

Quantum dots have been conjugated to HA backbone by first modifying HA with adipic acid dihydrazide followed by coupling the quantum dots to the carboxyl groups of HA through an amide linkage. They were then studied \textit{in vivo} for the effect of the chemical modification of HA on its cellular uptake and distribution. Real time imaging of the quantum dots was performed (they emit at 800nm) by injecting them either...
subcutaneously or in the vein of the tail of the mice. The distribution of the quantum dots was observed to depend on the amount of modification of the carboxyl group: the ones which were 35% derivatised had sufficient binding to HA receptors accumulated mainly in the liver (liver has high specificity towards HA molecules), while the ones which were 68% derivatised and had lost most of their binding sites were distributed evenly to the tissues in the body\textsuperscript{141}.

HA-poly(ethylene glycol)-poly(lactide-co-glycolide) [HA-g-PEG-PLGA]\textsuperscript{142} and HA-poly(ethylene glycol)-polycaprolactone [HA-g-PEG-PCL]\textsuperscript{143} nanoparticles have been prepared by Yadav et al. and have been tested \textit{in vivo} for biodistribution. The DOX loaded HA-g-PEG-PLGA and HA-g-PEG-PCL particles accumulated mainly in the liver and kidney will still quite substantial amounts localised in the tumour, whereas the free DOX was almost evenly distributed in the blood, heart, lung, liver, spleen and kidney. Control studies performed by substituting HA with methyl poly (ethylene glycol) showed that the particles delivered DOX to the tumour tissue much less efficiently than the HA\textsuperscript{142,143}.

Also, polycations such as poly(l-lysine) have been covalently grafted with short HA fragments for subsequent complexation with DNA for delivering DNA to the liver sinusoidal endothelial cells \textit{in vivo}\textsuperscript{144}.

Following the above examples, a HA-boronic derivative conjugated with a diol drug would also follow a similar pathway, whereby it would undergo receptor mediated endocytosis, followed by going through the early endosome where the receptor would get separated, followed by the late endosome and finally the lysosome where due to the acidic pH of 4.8, the drug would get released (boronic – diol links are unstable in acidic pH) and the HA-boronic would be degraded by the hyaluronidase enzymes.

1.2.6 Chemical Modifications in HA

\textbf{Principal Targets.} Hyaluronan, having limited biomechanical properties is not used for biomedical applications on its own. It is usually combined with other materials to improve its mechanical and chemical strength. Usually the non-toxicity and biodegradability of hyaluronan is retained after these chemical modifications. The carboxylic acid and hydroxyl groups are the two main groups which are used for chemical modifications in HA (Scheme 1-17). These modifications to HA allow it to be
used for various applications such as, space filling, drug delivery and tissue regeneration\textsuperscript{145}.

HA can be modified specifically for different purposes. If it is required to function as a gel or a hydrogel, it can be cross-linked\textsuperscript{146}. This makes it extremely viscous and elastic, thereby providing the properties of a gel. If it is required for drug delivery, tethering groups can be attached to hyaluronan which allow the attachment of drugs and their controlled release\textsuperscript{147}. Hyaluronan can be grafted onto other polymers to make composite biomaterials\textsuperscript{148}. It can also be grafted onto liposomes for drug delivery\textsuperscript{145}.

\section*{A) Modifications to carboxylic acid group}

There are two important reactions that can take place with the carboxylic acid group of HA: Esterification and Carbodiimide-mediated reactions\textsuperscript{145}.

\textbf{Esterification.} Esterification is a reaction in which carboxylic acids react with alcohols to form esters through a condensation reaction. Alkylation of tetra (n-butyl) ammonium salt of HA with an alkyl halide in DMF is one of the processes for preparing esterified hyaluronan biomaterials (Scheme 1-18). These materials have good mechanical strength when dry, but on combining with water they lose this strength. The degree of esterification plays an important role, whereby it varies the size of the hydrophobic part.
of the biomaterial. This hydrophobic part modifies the polymer network, making it strong and stable. Also it defers the degradation of the material by enzymes\textsuperscript{145, 149}.

\[
\text{HA-COON(BU}_4\text{) + RCH}_2\text{OH} \xrightarrow{\text{DMF}} \text{HA-CO}_2\text{CH}_2\text{R}
\]

**Scheme 1-18.** Esterification of tetra (n-butyl) ammonium salt of HA

Another common esterification reaction is the formation of HA-N-succinimide (HA-NHS) active ester (Scheme 1-19). This is an important intermediate for bioconjugation with reporter and effector molecules. Like the previous reaction, tetra (n-butyl) ammonium salt of HA reacts with N-hydroxysuccinimido diphenyl phosphate (SDPP) to form HA-N-succinimide active ester\textsuperscript{150}.

\[
\text{HA-COON(BU}_4\text{) + (PhO)}_2\text{P} \xrightarrow{\text{DMSO}} \text{HA-[C}_\text{O}_2\text{N]}_\text{N}
\]

**Scheme 1-19.** Esterification of HA to form HA-NHS active ester

A couple of HA-esterified biomaterials are commercially available (Scheme 1-20). These are known as HYAFF and are biomaterials with higher stability than pure HA and are produced by esterification of the free carboxyl group present in the HA chain with different alcohols\textsuperscript{149, 151}.

**Scheme 1-20.** Principal Structure of HYAFF Biomaterials

There are 2 variants present in this type of reaction – (i) using different alcohol groups (ii) changing the degree of esterification. These 2 variants ultimately lead to the
production of a variety of HYAFF biomaterials. HYAFF 7 and HYAFF 11 are two of the well known HYAFF biomaterials.\textsuperscript{151}

**Carbodiimide-mediated reactions.** Carbodiimide compounds react with carboxylic acid group of HA to produce carbodiimide-activated HA which can be used for derivatisation with drugs, for biochemical probes and for cross linking agents.\textsuperscript{145, 147, 152} Another use for carbodiimide-activated HA is hyaluronan microspheres.\textsuperscript{153} These have also been prepared using ADH and HA especially because HA possess structural integrity and has ease of removing unreacted reagents.\textsuperscript{154}

EDC [3-(dimethylamino)propyl] ethylcarbodiimide is one of the common carbodiimide compounds used to react with HA. Carbodiimide-mediated reactions might be performed with or without the use of primary amines. The additions of primary amine results in the production of O-acylisourea adduct. If there is no presence of nucleophile, then there is formation of stable N-acylurea, while in the presence of a nucleophile, the nucleophile attacks the O-acylisourea to form an amide linkage between the amide and the acid (Scheme 1-21).\textsuperscript{146, 152, 155}

![Scheme 1-21. Amidation of carboxylate group of HA](image)

Nucleophilicity is an important issue in carbodiimide-mediated reactions where the hydrazides need to retain their nucleophilicity in order to couple efficiently to carbodiimide-activated HA (Scheme 1-22). Hydrazides such as adipic dihydrazide (ADH) have more than one hydrazide groups which can be used for further reactions such as for derivatisation with drugs and for cross-linking agents.\textsuperscript{145, 156, 157}
Scheme 1-22. Carbodiimide-mediated reaction showing coupling of carboxylic acid group of HA with ADH to form carbodiimide-activated HA which further uses the free hydrazide groups to incorporate a drug.

The formation of carbodiimide-activated HA requires an acidic pH i.e. 4.75 because the amine base needs to have a certain percentage of itself in nucleophilic form for the reaction to occur successfully.\textsuperscript{145, 156}

The degree of derivatisation of HA can be varied depending on the nucleophile used. Also the conditions used for derivatisation should be mild in order to maintain high molecular weight of HA, since low molecular weight HA can cause inflammation.\textsuperscript{155}

B) Modifications to Hydroxyl group

There are four important reactions that can take place with the hydroxyl group of HA: (1) Esterification (2) Sulphation (3) Isourea coupling and (4) Periodate oxidations.\textsuperscript{145}

**Esterification.** Esterification, as mentioned before is the reaction between carboxylic acids and alcohols to form an ester. In this case, the hydroxyl group of HA is the alcohol and can react with different carboxylic acids to form various esters.\textsuperscript{145, 149, 158}

One of the common reactions is between butyric acid and the hydroxyl group of HA in the presence of DMF to form hyaluronan butyrate (Scheme 1-23). This HA butyrate can be used as a drug delivery system especially for tumour cells, since butyric acid is a well known compound which can stop cell differentiation in tumours.\textsuperscript{145, 158}
**Scheme 1-23.** Esterification reaction between hydroxyl group of HA and butyric acid to form HA butyrate.

**Sulphation.** Sulphation, in the simplest terms, can be explained as the addition of sulphur group to any chemical group. In the case of hyaluronan, the hydroxyl group can be reacted with sulphur trioxide-pyridine complex to give HASx where x can be anything from 1 to 4 per disaccharide (Scheme 1-24). X Represents the degree of sulphation. Also, this reaction is carried out in the presence of DMF\(^{145, 159-161}\).

**Scheme 1-24.** Reaction between hydroxyl group of HA and sulphur trioxide-pyridine complex to form sulphated HA.

Sulphated HA surfaces show less cell adherence and low bacterial growth as compared to native HA surfaces. These properties can be quiet helpful in certain applications like for medical devices.\(^{145}\)

**Isourea Coupling.** Isourea coupling is basically used for coupling antibiotic drugs to HA. Firstly the HA must be activated using cyanogen bromide. Only after the HA has been activated, can the drug be coupled with HA by urethane bonds\(^{145, 162, 163}\).

The reaction of the hydroxyl group with cyanogen bromide leads to the production of an isourea intermediate. This intermediate has a high affinity for antibiotic drugs and can couple with them easily (Scheme 1-25). The drugs attach via a urethane bond to one of the hydroxyl groups of the HA\(^{145, 162, 164}\).

The only drawback this process has is that this reaction can affect the integrity and biocompatibility of the HA\(^{145}\).
**Peridate oxidations.** Oxidation of the hydroxyl group of HA by sodium periodate results in the formation of periodate-activated HA. This periodate-activated HA can couple with primary amines and either cross-link, attach peptides to itself or immobilise materials (Scheme 1-26)\textsuperscript{145,165-167}.

\begin{align*}
\text{HA backbone} + \text{Br-C≡N} &\rightarrow \text{HA backbone} \\
\text{HC-O-C≡NH} + \text{drug-NH}_2 \\
\text{HC-O-CONH-drug} \\
\text{HA backbone}
\end{align*}

**Scheme 1-25.** Activation of HA by cyanogen bromide and isourea coupling of drugs.

This is a 2 step process in which the HA is first activated and then by the process of reductive coupling, primary amines are attached to HA. The drawback of this process is that this oxidation technique might break the chain and introduce immunogenic linkages into the HA chain\textsuperscript{145,166}.
C) Crosslinking techniques

Hyaluronan has 4 reactive groups (carboxyl, hydroxyl, reducing end and amide) which participate in crosslinking of HA either with itself or other polymers\textsuperscript{168}.

The crosslinking techniques basically involve the modifications used above, but are aimed for the purpose of achieving a mechanically strong material\textsuperscript{145}.

Various experiments have suggested that a high degree of crosslinking and therefore high stability can be achieved by carrying out the process of crosslinking in 2 stages. In both the stages the same crosslinking agent is used, however the different functional groups are used by altering the reaction conditions\textsuperscript{168}.

Some of the crosslinking techniques are described below:

\textbf{Carbodiimide crosslinking.} Carbodiimide’s react with carboxyl group to form an unstable O-acylisourea, which in the absence of a nucleophilic agent forms N-acylurea which is stable. If a primary amine is introduced, it acts as a nucleophilic agent, and the O-acylisourea does not form N-acylurea and instead leads to the formation of an amide linkage between the amine and the acid. This is a desirable product in the case of crosslinking. In this reaction, amide linkages are formed which couple to the HA which make the substance chemically stable. Also, there are no undesirable side-products formed\textsuperscript{145, 146}.

The carboxyl group of HA reacts with a biscarbodiimide in aqueous isopropanol to form carbodiimide crosslinked HA (Scheme 1-27). This leads to the production of a low water content hydrogel. Hyaluronan films can also be made using carbodiimide’s in the same way\textsuperscript{145, 146}.

The chemical properties of this type of cross-linked HA depends on the ease of availability of the carboxyl groups to the carbodiimide’s and not merely on its functional groups\textsuperscript{146}.

\begin{center}
\textbf{Scheme 1-27.} Crosslinking of HA using carbodiimide
\end{center}
Hydrazide crosslinking. HA can be cross-linked using hydrazide compounds such as bishydrazide, trishydrazide and polyvalent hydrazide. HA-ADH which is formed by reaction of adipic dihydrazide with carboxylic group of HA can be cross-linked using small molecule homobifunctional cross-linkers such as disuccinimidyl suberate (DSS) (Scheme 1-28)\(^{145,169}\).

HA-ADH can be also be cross-linked using EDC. This cross-linked HA can be used as a drug delivery vehicle. HA-ADH can also be cross-linked for hydrocortisone release using hydrocortisone hemisuccinate solution\(^{147,154}\).

The gels formed from this technique can range from soft gels to rigid gels. The mechanical properties of the gels can be varied, by changing the reaction conditions and molar ratios of the reactants\(^{145,147,170}\).

PEG cross-linkers and PVA cross-linkers are also used for making gels. Experiments have shown that PVA cross-linkers have better gelation capacity than PEG cross-linkers since PVA is polyfunctional and PEG is just bifunctional\(^{171}\).

\[2\text{HA} + \text{Disuccinimidyl suberate (DSS)} \rightarrow \text{HA-ADH} + \text{PEG} \]

**Scheme 1-28.** Crosslinking of HA using hydrazide

HA-ADH can also be cross-linked with PEG-dialdehyde, which is a macromolecular cross-linker (Scheme 1-29). This leads to the formation of a hydrogel with considerable strength\(^{145,169}\).
PEG can also be grafted on HA and be used for drug delivery. This leads to the formation of an aqueous polymer two phase system which can incorporate bioactive peptides like insulin. When a peptide such as insulin is inserted in pure PEG, it gets entangled with the PEG, however in this case when PEG is grafted onto HA, it remains separate. This type of system can be used for injectable formulations\textsuperscript{172}.  

\textbf{Scheme 1-29.} Crosslinking of HA using PEG-dialdehyde
1.3 Biological relevance of catechols

1.3.1 Catecholamines

Catecholamines are sympathomimetic “fight-or-flight” hormones secreted by the adrenal glands in response to stress and belong to the sympathetic nervous system. They are derived from the amino acid l-tyrosine and contain a catechol or 3,4-dihydroxylphenyl group and an ethylamine moiety. The main catecholamines which circulate in the blood but cannot cross the blood-brain-barrier are epinephrine (adrenaline), norepinephrine (noradrenalin) and dopamine (Scheme 1-30)\textsuperscript{173,174}.

![Structure of catecholamines](image)

\textbf{Scheme 1-30.} Structure of catecholamines

\textbf{Dopamine.} Dopamine is a neurotransmitter bridging the nervous and immune systems. Dopaminergic neurons mainly exist in the mid-brain and partly in the hypothalamus\textsuperscript{175}. It regulates behaviour, movement, endocrine, cardiovascular, gastrointestinal, renal, and immune functions\textsuperscript{176-178}.

Dopamine acts through 5 main G-protein coupled receptors D\textsubscript{1}, D\textsubscript{2}, D\textsubscript{3}, D\textsubscript{4} and D\textsubscript{5} in normal human leukocytes. D\textsubscript{1} and D\textsubscript{5} receptors are classified as excitatory binding (D\textsubscript{1} type, positively coupled to adenylate cyclase) while D\textsubscript{2}, D\textsubscript{3} and D\textsubscript{4} are classified as inhibitory binding (D\textsubscript{2} type, negatively coupled to adenylate cyclase). Human dendritic cells and lymphocytes have shown to contain dopamine receptors as well. Changes in the expression of dopamine receptors and their signalling pathways alter immune functions in disorders such as schizophrenia and Parkinson’s disease\textsuperscript{176-179}. 
Dopamine is generated by a 2 step conversion: firstly L-tyrosine is hydroxylated to L-dopa by the enzyme tyrosine hydroxylase followed by decarboxylation of L-dopa to dopamine by the enzyme L-amino acid decarboxylase.\(^{173-175}\) Dopamine can be formed by hydroxylation of endogenous neurochemical substrate tyramine by CYP2D6 (a CYP2D isoform). CYP2D belongs to the most important cytochrome family which is capable of metabolizing various drugs such as antidepressants, neuroleptics, antiarrhythmic agents, the drugs of abuse – codeine and amphetamine, carcinogens and various neurotoxins.\(^{180}\) CYP2D are also present in the brain and regulate the variations in response to centrally acting drugs and toxins. Since dopamine cannot cross the blood-brain-barrier, CYP2D6 synthesises dopamine from tyramine in the liver as well as in the brain separately.\(^{175, 180}\) Dopamine has been shown to form strong bonds with various organic/inorganic surfaces and hence self-polymerisation of dopamine is a useful way for applying multifunctional coatings onto surfaces like noble metals, metal oxides, ceramics and polymers.\(^{181}\) One example of this is the report by Park where dopamine-derivatised heparin was anchored on the surface of a cobalt-chromium alloy along with paclitaxel encapsulated in HA-g-PLGA micelles and poly-L-lysine to form a drug-eluting stent for coronary artery disease.\(^{182}\) Another example is shown by Messersmith where polydopamine surfaces were engineered for specific biomolecular interactions by forming an ad-layer of partially thiolated hyaluronic acid. Dopamine has also been incorporated in intracranial nano-enabled scaffold devices for site-specific delivery to minimize the side-effects of conventional forms of Parkinson’s disease therapy.\(^{184}\) Levodopa, a precursor of dopamine has also been used in minitablets with extended release times.\(^{185}\) Also, HA-dopamine mixed with thiol end-capped Pluronic F-127 copolymer has been used to produce a lightly cross-linked composite temperature dependant gel for drug delivery.\(^{186}\)

**Epinephrine.** Epinephrine is localized in the adrenal medulla and in the brain.\(^{187, 188}\) Epinephrine is synthesised from norepinephrine by phenylethanolamine N-methyltransferase in the brain.\(^{188-191}\) Epinephrine acts by binding non-selectively to various adrenergic receptors like \(\alpha_1, \alpha_2, \beta_1, \beta_2\) and \(\beta_3\) receptors. Binding to \(\alpha\) receptors inhibits insulin secretion by pancreas, stimulates glycogenolysis in liver and muscle, and starts glycolysis in muscle. Binding to \(\beta\) receptors triggers glucagon secretion in pancreas, increases adrenocorticotropic
hormone secretion by the pituitary gland, and increases lipolysis by adipose tissue. This leads to increased blood glucose and fatty acids for energy production within cells\textsuperscript{192, 193}.

It is released during sudden frightening or life-threatening situations and affects sympathomimetically innervated structures all across the body. The heart rate rises increasing the force of cardiac contractions and increasing the blood pressure. The blood flow is also redistributed from the skin and subcutaneous tissue to the skeletal muscles, splanchnic circulation, and brain. The bronchi and pupils also dilate with increase in oxygenation and blood glucose to ‘fight or flight’\textsuperscript{187, 193}.

Epinephrine is metabolized by catechol-O-methyl transferase (COMT - ubiquitously expressed enzyme that metabolizes catecholamines) to produce metanephrine in the presence of the cofactor S-adenosylmethionine\textsuperscript{194-196}.

Epinephrine is used for treatment of postintubation croup and viral croup by face masks and nebulizers. Dilute solutions of epinephrine are used in surgery to provide hemostasis and/or prolong the duration of action of concomitantly administered local anesthetics. It is also largely used for treatment of anaphylaxis\textsuperscript{187, 195} and septic shock\textsuperscript{197}.

Epinephrine has also been encapsulated in alginate based microspheres\textsuperscript{198} and coloaded in biocompatible polymer implants\textsuperscript{199} to ensure vasoconstriction at delivery at various rates.

**Norepinephrine.** Norepinephrine is a major monoamine neurotransmitter which is synthesised in both the adrenal medulla and in the brain\textsuperscript{189, 200, 201}. Mainly it regulates arousal and stress responses and any dysregulation with this system leads to anxiety and depressive disorders\textsuperscript{202}. Norepinephrine also regulates various other processes such as decision-making, attention response, sleep, memory encoding, and fear-learning\textsuperscript{203, 204}.

The main norepinephrine receptors are the same as epinephrine receptors $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$ and $\beta_3$ which carry out its modulatory activities\textsuperscript{203, 205}.

Norepinephrine is synthesised in the brain by hydroxylation of dopamine (using enzyme dopamine-β-hydroxylase) which is synthesised by decarboxylation of dihydroxyphenylalanine (using enzyme dopa decarboxylase) which is synthesised from tyrosine (using enzyme tyrosine hydroxylase)\textsuperscript{201, 206, 207}.

The metabolism of norepinephrine in humans could be either: o-methylation which forms normetanephrine, deamination by monoamine oxidase which forms dihydroxyphenylethylene glycol (DHPG) and dihydroxymandelic acid (DOMA), or
combined deaminition and o-methylation by catechol-O-methyltransferase which forms vanillylmandelic acid and 3-methoxy-4-hydroxyphenylglycol\textsuperscript{200, 205}. Norepinephrine is mainly used in reversing hypotension in patients with septic shock as it is more effective than dopamine\textsuperscript{208}. It has also been used in spinal injections along with opioids for reducing chronic pain\textsuperscript{209} and in biodegradable microspheres for treatment of CNS neurodegenerative disease\textsuperscript{210}.

### 1.3.2 Other catechols (2- and 4-hydroxyestradiol)

Estradiol, the primary female sex hormone responsible for sexual differentiation and development, arterial vasodilation, maintenance of bone density and neuroprotective actions is eliminated from the body by metabolic conversion to inactive metabolites that are excreted in the urine and/or feces\textsuperscript{211}. Estradiol is converted to 2- and 4-hydroxyestradiol (Scheme 1-31) in the endoplasmic reticulum by NADPH-dependent (nicotinamide adenine dinucleotide phosphate dependent) cytochrome P450-linked mono-oxygenase system\textsuperscript{212-214}. Conversion to 2-hydroxyestradiol is carried out by CYP450 1A1/1A2/3A4 whereas to 4-hydroxyestradiol is carried out by CYP450 1B1\textsuperscript{211, 213}.

![Scheme 1-31. Metabolism of estradiol to 2- and 4-hydroxyestradiol by CYP450.](image)

Unconjugated 2-hydroxyestradiol concentration is low in blood and several tissues because of its instability and its rapid O-methylation by COMT to form 2-
methoxyestradiol which has negligible estradiol receptor binding affinity and targets actively dividing cells disturbing cell proliferation (antitumourigenic)\textsuperscript{211, 213-215}. It has been shown that catechol metabolites of estradiol (mainly 4-hydroxyestradiol) may lead to carcinogenesis possibly due to their rapid oxidation leading to production of quinines and reactive oxygen species which can lead to deoxyribonucleic acid damage and mutations. This has been shown in the study of breast cancer\textsuperscript{211, 213, 214, 216}. Catecholestrogens have been proved to be better antioxidants than estradiol\textsuperscript{217, 218}. The administration of 2-hydroxyestradiol has shown to suppress the increase in lipid peroxide levels caused by ovariectomy in women. This capability of 2-hydroxyestradiol could also be used to prevent cardiovascular disease\textsuperscript{217, 219, 220}.

1.3.3 Flavonoids (Quercetin, Taxifolin, etc.)

Flavonoids are polyphenolic compounds found in common foods we eat. Over 4000 different ones have been identified, the major sources being apples, berries, onions, chocolate, red fruits, citrus fruits, nuts, and drinks such as beer, tea and wine\textsuperscript{221-223}. The basic structure of flavonoids consists of a heterocyclic ring fused to an aromatic ring, attached to a third ring system at C3 or C4 position\textsuperscript{224, 225}. Some of the most common catechol containing flavonoids are quercetin, taxifolin, myricetin, fisetin, luteolin and epicatechin (Scheme 1-32)\textsuperscript{223}. Quercetin is one of the main flavonoids as it is present abundantly in onions and apples and it is proven to prevent diseases associated with oxidative stress such as cancer and cardiovascular diseases\textsuperscript{224-226}.

Flavonoids have a positive influence on various cardiovascular diseases due to their antioxidant activity, metal interactions (iron and/or copper chelation), decrease in expression of inflammatory signaling molecules, inhibition of platelet aggregation, vasodilatory action and their capability to inhibit radical-forming enzymes like NADPH oxidase, xanthine oxidase and lipoxygenases\textsuperscript{221, 223, 224, 227}.

In the brain, they have a potential to protect neurons against injury by neurotoxins, suppress neuroinflammation, and promote memory, learning and cognitive functions\textsuperscript{228}. 
Flavonoids have been described as CYP1 inhibitors due to their inhibition potential of carcinogenic product formation and blockage of the initiation stage of carcinogenesis (over expression of CYP1 in tumours). However they are also responsible for CYP1-mediated bioactivation, for example; kaempferol is a inhibitor of CYP1A1 but, in doing so, it is transformed to quercetin. Some of the flavonoids like quercetin have been encapsulated in nanoparticles, microcapsules, vesicles and micelles for anticancer drug delivery. Taxifolin has also been used for its anticancer, antioxidant and anti-inflammatory properties by encapsulation in polyvinylpirrolidone nanoparticles. Troxerutin gels have also been synthesised for topical treatments as antioxidants, and as protective agents on the wall of veins for prevention of varicose veins.

Scheme 1-32. Catechol containing flavonoids.
1.4 Scope of this thesis

In the current investigation we aim at: a) Studying the pH based reversible complexation of boronic acids with diols using the batochromic shifts of the reporter alizarin red S (ARS) in simple and complex systems. This is purely a new analytical method to evaluate their binding constants and to give an idea of how easy/difficult would it be to release a diol. b) Studying the synthesis, characterisation, cytotoxicity and degradability of a HA-boronic based drug delivery carrier and other HA derivatives. c) Studying the reversible complexation of HA-boronic with diols using batochromic shifts of ARS in simple and complex systems and to evaluate the effects/advantages of using a polymeric chain. d) Studying the bioconjugation of HA-boronic with diols and vice versa using static light scattering.

Bioconjugation has several advantages, however the few constraints which need to be focussed on for good bioconjugate development are: a) To have the perfect conditions for binding so as not to affect the stability of the polymer. b) To ensure adequate and successful detachment of the drug at the target site in endosomes and lysosomes. c) To develop methods for characterisation of the constructs as a whole. d) To obtain a well-characterised biodegradable polymeric carrier. e) To choose a polymer which can be easily approved by the FDA.

In Chapter 2 we have presented the study of complexation of boronic acids with various diols using the batochromic shifts of the optical reporter ARS. Since the complexation of polymeric compounds with diols is more complicated, we have initially analyzed the equilibrium constants of the reactions between 3-aminophenylboronic acid and various diols (catecholamines, sugars, flavonoids, etc.) in order to test the feasibility of the new analytical technique as well as to establish a benchmark for the binding constants which can later be used to compare with those obtained by using polymeric compounds. The main advantage of using this method over literature methods of using fluorescence is that it can be employed to complex systems such as colloidal dispersions or complexed active compounds. In order to evaluate the other uses and advantages of this method, we have also studied the action of enzymes on catechols, focussing on cytochrome P450-mediated reactions which could be used as a reporter for these reactions in a biological environment. One such reaction studied is the conversion of hormones such as cyclodextrin-complexed estradiol to 2-hydroxyestradiol using
CYP1A2. The other reaction studied is the demethylation of 3-methoxytyramine using CYP2D6 to dopamine which is a major drug for Parkinson’s disease.

**Scheme 1-33. Design of a HA based responsive polymeric carrier**

In Chapter 3 we have presented the synthesis, characterisation, cytotoxicity and degradability studies of three HA derivatives (HA-boronic, -dopamine and -veratrylamine) synthesised using an amidation reaction. We have also studied the reaction and its side effects in detail. We have specifically focused on HA-boronic since it appears to be a suitable drug carrier, due to its low toxicity and the complexing ability towards diol-containing molecules (Scheme 1-33). We have therefore studied the complexation of HA-boronic with the same library of diols (soluble, in colloidal form, enzymatically generated) as in Chapter 2 and have provided a comparison. In addition we have also studied the complexation of HA-boronic with other polymers (HA-dopamine, PEG-dopamine, etc) in order to evaluate the binding of HA-boronic with large molecules. Future work will establish whether HA-boronic diol-containing-drug complexes will be able to release the drug selectively in the acidic environment of the late endosome or lysosome.
1.5 References

49. Hein, J. E.; Fokin, V. V. Chemical Society Reviews 2010, 39, (4), 1302-1315.
70. Bendel, P. *Nmr in Biomedicine* 2005, 18, (2), 74-82.
Chapter 1


2. New tricks for an old dog: boronic-diol “click” reactions in complex media. Applications to enzyme kinetics and catecholamine metabolism
Chapter 2

2.1 Summary

The complexation of boronic acids and diols is an interesting “click” reaction, used both in natural and synthetic systems. These reactions are often monitored through the fluorescence developed by Alizarin Red S upon complexation with boron derivatives. We here describe an alternative method based on the batochromic shifts of this reporter; the distinct advantage of this method is the possibility to be applied also to complex systems, such as colloidal dispersions or complexed active compounds. This is purely a new analytical method to study the conjugation between boronic acids and diols by evaluating their binding constants and to give an idea of how easy/difficult would it be to release the diol. To evaluate the new analytical method we have successfully employed the batochromic shifts of ARS to the determination of the equilibrium constants of the reactions between a number of diols, several with catecholamine structure, and a model boronic derivative. Since the complexation of polymeric compounds with diols is more complicated, we have initially followed the complexation of a small molecule (APBA) with diols in order to test the feasibility of the new analytical technique as well as to establish a benchmark for the binding constants which can later be used to compare with those obtained by using polymeric compounds. Most interestingly, in order to present the other advantages and uses of this method, we have showed that this method allowed to follow the kinetics of enzymatic reactions involving catechol structures; we have focused on cytochrome P450-mediated reactions and, besides demonstrating the possibility to follow enzymatic modification on complexed steroids (cyclodextrin-complexed estradiol→2-hydroxyestradiol), we have for the first time shown the enzymatic (CYP2D6) reversibility of dopamine methylation, which is a catechol O-methyltransferase (COMT)-mediated process presiding to the inactivation of drugs for Parkinson’s disease (PD). This last result may open new perspectives in PD treatment, where both COMT and CYP2D6 polymorphism may have to be taken into account.
Chapter 2

2.2 Introduction

2.2.1 Significance of boron-diol complexation

Boric acid and organic boronic derivatives are well known to interact with diol-containing molecules with high affinity, reversibly forming cyclic esters in a sort of “click” reaction, orthogonal to most biochemical pathways. These reactions occur in nature: in the plant or fungal world such complexes are found in cell walls\(^1\) and have an important function in the stabilization of plant cell membranes\(^2\), and have also been shown to mediate the biological mobility of boron\(^3\). Another interesting example of the biological role of boron-diol (catechol) complexes is offered by certain bacterial quorum-sensing molecules\(^4\)-\(^6\), whose activity can be modulated or inhibited by the formation of complexes with boronic/boric acid, then affecting bacterial virulence and capacity of swarming or of forming biofilms\(^6\),\(^7\).

These reactions are also selective: due to their preferential interaction with 1,2 diols in a chelating configuration, and specifically with \textit{cis} sugars, boronates have been proposed as synthetic analogues of lectins, as suggested by Wang\(^8\),\(^9\). Due to this selective recognition of carbohydrates, as reviewed by Striegler\(^10\), boron-based sugar sensors have been developed, for example conjugating boronic acids with fluorescent reporters\(^11\),\(^12\). Boronate-displaying solid supports have also been used for the purification/extraction and analysis (chromatography\(^13\)-\(^15\), electrophoresis\(^16\),\(^17\)) of carbohydrate-containing low MW or polymeric compounds, such as glycoproteins; the reverse approach has also been employed, and supported diols have been used for the extraction of boronic acids\(^18\) or for performing solid state chemistry on them\(^19\).

In synthetic polymer chemistry, the boronate/diol complexation has been used as a “click” reaction, e.g. to provide functional ends\(^20\), or to fine tune the nano-structure of self-assembling macromolecules\(^21\); this reaction has also been employed as a reversible polycondensation reaction\(^22\), e.g. to provide self-repairing macromolecules\(^23\). Finally, and most interestingly, the mild conditions of boronate/diol complexation and its insensitivity to most biomolecules has also allowed its use for the derivatisation of biological surfaces, such as those of living cells\(^24\),\(^25\).
2.2.2 Details of boron/diol complexation

Following complex formation, boron is readily quaternarised by OH ions, leading to a pH decrease\(^{26}\), *inter alia*, this phenomenon has been extensively used for the titration of \(\text{H}_3\text{BO}_3\) with bases\(^{27, 28}\), otherwise much too weak an acid. However, since the free boronates can be quaternarised by OH ions too, one can consider two different constants for the boronate/diol complexation: one \(K_{\text{trig}}\) for the reaction from the trigonal form \((\text{R-B(OH)}_2)\) and one \(K_{\text{tet}}\) for the tetrahedric, quaternarised form \((\text{R-B(OH)}_3^-)\). However, the higher acidity of the complex causes \(K_{\text{tet}} \gg K_{\text{trig}}\), and it is therefore possible to group the formation of the cyclic product and its quaternarisation into one single complexation event. The acidity and therefore also the \(K_{\text{tet}}\) of the boron/diol complex appears not to have a strong dependence on the pKa of the boronic precursor\(^{30}\), but it does depend on that of the diol; for example cathecols are more acidic than aliphatic alcohols and bind more strongly than them. There are exceptions to this rule: the binding constants of \(\alpha\)-hydroxyacids to boronic acids are only marginally higher than those of aliphatic diols\(^{31}\).

A variety of methods have been employed for the quantification of boronic/diol equilibria. In the vast majority, they exploit spectral changes produced by the complexation/decomplexation of a chromophoric reagent, such as electron-rich (hyperbranched) polyphenylenes\(^{32}\) or naphtyl residues\(^{33, 34}\). Probably the most effective and popular reporter is alizarin red S (ARS), an aromatic diol capable of intense fluorescence only when bound to a boronic acid\(^{20}\). If a second diol is added, two competing equilibria are established (Scheme 2-1): in the first one the ARS reacts with the boronic acid, in our case 3-aminophenyl boronic acid (APBA) as a model of functional boronic acids. Upon addition of another diol (DI), an APBA – DI adduct can be formed, with the contemporaneous liberation of ARS. A lot of literature in the past has been focused on the use of phenylboronic acids with ARS since they are water soluble and can easily complex with diols at neutral pH due to their pK\(_a\)\(^{8, 9}\). However, in this study we have specifically used APBA since its amine group can be linked with the carboxylate group of HA to form HA-BA which could be used as a carrier for diol based drugs. So, in order to evaluate the complexation between HA-BA and diols, a benchmark using the new analytical technique was required, which was provided by studying the complexation between APBA and various diols. Knowing the equilibrium
constant for the first equilibrium ($K_{ARS}$) and monitoring the concentration of ARS, it is possible to calculate the second equilibrium constant ($K_{Diol}$). Due to their favourable characteristics, these equilibria have also been employed to build chemical gates, linking saccharide sensing to molecular computing\textsuperscript{35}.

![Scheme 2-1](image)

**Scheme 2-1.** The spectral changes of ARS allow to evaluate the equilibrium constants a) of its binding to a model functional boronic acid, APBA, ($K_{ARS}$) and b) of the competitive binding to APBA in the presence of another diol ($K_{EXC}$). From these constants, it is possible to calculate that of the equilibrium between the diol and APBA ($K_{Diol}$). Please realize that for simplicity the protonation equilibria have been omitted and all free boronic acids have been represented in their protonated form, their complexes in their deprotonated one.

### 2.2.3 Validation of the analytical method

In this study, we have developed a parallel method based on the changes in the ARS absorption spectrum: a peak shift of about 40 nm is observed using 0.56 mM ARS and 0.56 mM APBA (519 nm – 480 nm) i.e. a colour change from red to orange is associated to complex formation, while the liberation of free ARS determines a colour shift back to the red region. This absorbance-based method is considerably less sensitive than any fluorescence-based assay, but is applicable to far more concentrated and possibly inhomogeneous samples and is also much less sensitive to the presence of
scattering objects and quenchers. The absorbance method, therefore, would be suitable to follow diol/boronic equilibria used as “click” reactions in complex, e.g. heterogeneous or self-assembled systems, where scattering or aggregation (self-quenching) may provide unreliable fluorescence readings. Here, we have first validated the use of absorbance for the evaluation of the constants for the equilibria between APBA and a number of aromatic diols (catechols) and aliphatic ones (monosaccharides), which are listed in the Scheme 2-2.

The main reasons for using these diols are to prove the usability of the analytical technique with various diols, to compare the binding constants between similar types of diols (catechols, aromatic polyols, aromatic negative controls and sugar diols) and to investigate whether the different positioning of diols in various substances has any effects on their binding constants.

<table>
<thead>
<tr>
<th>(Precursors)</th>
<th>Aromatic diols (catechols)</th>
<th>Aromatic polyols</th>
<th>Aromatic negative controls</th>
<th>Sugar diols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrocatechol</td>
<td>Pyrogallol</td>
<td>Phloroglucinol</td>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>3-methoxytyramine</td>
<td>Dopamine</td>
<td>Veratrylamine</td>
<td>Mannose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Noradrenaline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epinephrine (Adrenaline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>2-Hydroxy estradiol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

_Scheme 2-2._ Structures of diols used in this study.
Another important reason for using these substrates is to provide a benchmark value of binding constant for each substrate with APBA so as to be able to compare with the binding values for HA-3-APBA which would help in determining any effect a polymer would have on the binding strengths and hence on the ease of binding and release of these diols – thereby contemplating on the use of a boronate – polymer as a drug delivery carrier.

Pyrocatechol, dopamine, norepinephrine and epinephrine present a simple 1,2-dihydroxybenzene structure and have been chosen as main substrates; it is noteworthy that the last two are metabolites of dopamine. The study of dopamine is also carried out since we expect HA-3-APBA and HA-Dopamine when mixed to form pH responsive nanoparticles which could be used for the delivery of hydrophobic drugs to cancer/tumour cells. Pyrogallol has been used as a positive control: it bears an additional OH group that can speed up the kinetics of complexation (two positions of attack) but it should not dramatically influence its equilibrium constant. As negative controls, i.e. non-complexing but structurally similar reagents, we have used phloroglucinol, where the spacing between OH groups does not allow the formation of cyclic esters, and veratrylamine, a dimethylated analogous to dopamine. Finally, as “sugar” diols we have used glucose and mannose, and ascorbic acid, an enediol derived from glucose.

We have then employed this assay to equilibria occurring in complex and possibly phase-separated environments:

**Colloidal dispersions.** Flavonoids have attracted attention as promising active principles in the prevention of cancer\(^3^6\),\(^3^7\) and inflammation\(^3^8\), possibly due to the radical scavenging and reducing\(^3^9\) activity of their catechol-containing structure. Quercetin is the most common naturally occurring flavonoid and possibly the most investigated one, but its scarce water solubility\(^4^0\) determines the need of solubilisers, which on the other hand may affect its physico-chemical properties. Here we have used a surfactant already known to effectively disperse quercetin, Tween 20\(^4^1\), verifying the availability of quercetin’s catechol group through the absorbance test. The main reason is to prove the usability and advantage of this method for determining the binding constants of hydrophobic substrates containing diols with boronic acids as most of the anti-tumour/cancer drugs are usually hydrophobic.
**Chapter 2**

*In situ enzymatically produced substrates.* A number of metabolic pathways involve catechols and the availability of a reporter to continuously monitor these reactions in a biological and possible heterogeneous environment would be very beneficial. We have used two different enzymatic pathways to prove this and it was carried out to purely present the various instances in which this method might be useful, especially for monitoring the metabolic effects of CYPs on drugs. We have specifically focused on the human Cytochrome P450 enzymes (CYPs), a vast family of enzymes whose very wide metabolic activity is directed to both endogenous and exogenous molecules. CYPs are best known for their oxidative activity, which is often essential for drug metabolism and makes them one of the most important targets of rational drug design.

Four main classes of chemical reactions are typically performed by CYPs, i.e. carbon hydroxylation, heteroatom release, heteroatom oxygenation and epoxidation.

**A)** The conversion of phenolic substrates to catechols belongs to the first class of reactions (hydroxylation) and is often related to tumour initiation and progression: for example, the CYP-mediated oxidation of estrogens to the corresponding catechols and further to quinonic structures are well established steps in hormonal cancerogenesis. Here we have focused on estradiol (17β-estradiol) hydroxylation, a reaction whose regiochemistry is critical in tumour development: the 2-hydroxy derivative has a catechol structure and is known to be non-cancerogenic and possibly tumour-inhibiting because of its rapid monomethylation by catechol O-methyltransferase (COMT); the 4-hydroxy derivative has a much richer REDOX chemistry and high tumourigenic activity. Our method may allow to selectively follow the production of the 2-derivative, allowing to compare possibly tumourigenic conditions. Here we have employed the P450 variant 1A2 (CYP1A2), one of the three variants known to operate the 2-hydroxylation reaction. Since both estradiol and 2-hydroxyestradiol, are substantially water insoluble, we have used a sulphonated β-cyclodextrin to solubilise them. Cyclodextrins are good complexing agents for estradiol, even inhibiting its hormonal activity that is based on its hydrophobic character; on the other hand, compared to most common excipients, they have a very low inhibitory activity on CYPs. We have chosen a sulphonated cyclodextrin because preliminary experiments have showed that it allows for a higher maximal concentration of estradiol, while the sulphonation of OH groups reduces the interference of the cyclodextrin diols in the complexation of APBA.
B) Some members of the CYP superfamily are specifically active in performing the second class of reactions (heteratom release) by demethylating or demethylenating phenolic and catecholic substrates: for example, cytochrome P450 variant 2D6 (CYP2D6), which is estimated to be responsible for the metabolism of at least a quarter of the drugs used today\(^{53}\), has a significant O-demethylase activity. CYP2D6 appears to be the main enzyme responsible for the dealkylation of methylated phenols yielding, e.g. serotonin (from 5-methoxytryptamine)\(^{54}\), morphine (from codein)\(^{55}\), desvenlafaxine (from venlafaxine, a serotonin-norepinephrine reuptake inhibitor)\(^{56}\), harmalol and harmol (respectively from harmalin and harmin, two psychoactive alkaloids inhibiting monoamine oxidase A)\(^{57}\), dextorphan (from dextromethorphan, a cough suppressant)\(^{58}\), O-desmethyl-tramadol (from tramadol, a common analgesic)\(^{59}\). CYP2D6 exhibits a similar dealkylating activity on methylene dioxolane catechol derivatives (e.g. 3,4-methylenedioxyamphetamine (MDMA, ecstasy))\(^{60}\) converting them into catechols. Although this enzyme can also perform hydroxylation reactions on phenolic substrates, converting them to catechols (for example \(p\)- or \(m\)-tyramine to dopamine\(^{61}\), in the presence of methyl ethers it has predominantly a dealkylase activity\(^{62}\) and hydroxylation is only a less likely second step catalyzed by the same enzyme\(^{63}\). Due to its demethylating activity, CYP2D6 may help countering the role of COMT in the therapy of Parkinson’s disease (PD): COMT is one of the main contributors to the inactivation of exogeneous neurotransmitters, such as L-DOPA, dopamine, epinephrine, etc, used to treat PD\(^{64, 65}\). As a result, COMT inhibitors (entacapone, tolcapone, etc.) are now used in combined therapy with L-DOPA to increase dopamine concentration in the brain\(^{66-68}\). CYPD26 may partially counter COMT action, for example demethylating 3-methoxytyramine, which is the mono-methylated derivative of dopamine produced by COMT; however, we have not found any report in the literature about this reaction. We have therefore applied our batochromic shifts method to investigate whether CYP2D6 could produce dopamine from 3-methoxytyramine.
2.3 Experimental Section

2.3.1 Materials

Alizarin red S (ARS), 3-aminophenylboronic acid hydrochloride (APBA), phenylboronic acid (PBA), dopamine hydrochloride (DOPA), D-(-)-mannose, D-(-)-glucose, pyrogallol, phloroglucinol, L-ascorbic acid, quercetin, estradiol, pyrocatechol, (±)-epinephrine hydrochloride, D,L-norepinephrine, veratrylamine, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), recombinant human cytochrome P450 1A2 (1000 picomol/mL solution), Tween 20, sodium hydroxide pellets and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were supplied as high purity reagents (purity always ≥ 98%) by Sigma-Aldrich (U.K.) and used without further purification.

Sodium dihydrogen orthophosphate dihydrate, and disodium hydrogen orthophosphate dihydrate were supplied by BDH (U.K.). Human Cytochrome P450 2D6 Yeast reductase (500 picomol/mL solution) was manufactured by SPI-Bio (France) and supplied by Immuno diagnostic systems (U.K.). β-cyclodextrin sulphobutylether (Captisol) was supplied by Cydex Pharmaceuticals (U.S.).

All solutions were prepared in 100 mM PBS obtained by dissolving sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate dihydrate and sodium chloride, supplied by BDH (U.K.), in concentrations respectively of 2.3 g/L, 11.8 g/L and 9 g/L in water purified in a Milli-Q system (Millipore, U.K.).

2.3.2 UV-Vis and fluorescence measurements

A BioTek Synergy 2 multi-mode microplate reader was employed to record absorbance spectra (generally in the range λ = 400-800 nm) and fluorescence readings (filters at λ\text{exc} = 485±20 nm and λ\text{em} = 620±40 nm); temperature was generally kept at 25°C, except for the spectra of enzymatic reactions which were recorded at 37°C. All the spectra were corrected by subtracting the possible scattering component of the buffer solution using appropriate blanks.
2.3.3 Binding experiments

**General conditions:** For competitive binding experiments in each well of a 96 well plate (total volume of each sample = 250 µL) 48 µL of a 1 mg/mL ARS solution and 55 µL of a 0.44 mg/mL APBA solution (both in 0.1 M PBS) were mixed to obtain a 0.56 mM final concentration for both reagents and were allowed to react for 30 seconds; the colour of the solution correspondingly changed from red to orange. An amount of diol corresponding to 3-APBA/diol molar ratios ranging from 1:0.5 to 1:1000 was then added, producing a clear orange to red chromatic change at high 3-ABPA/diol molar ratios. The highest APBA:diol molar ratio for veratrylamine and for phloroglucinol was 1:10 (0.0056 M) and 1:100 (0.056 M) respectively, due to their limited solubility in water. All the absorbance values and the concentrations of the reagents were then corrected for the dilution. In simple binding experiments the same protocol was used, but the volumes of APBA or PBA solutions were varied in a series of experiments and the absorbance or fluorescence values were monitored to obtain the intensities as a function of amount of added boronic acid.

**Reaction with pyrogallol:** Owing to the quick oxidation of this electron-rich polyol, TCEP was added to the stock solution of pyrogallol in a molar ratio pyrogallol/TCEP 1:1.5. Due to the decrease in pH following TCEP addition, a few drops of 0.1 N NaOH were added to the pyrogallol stock solution until pH = 7.4.

**Reaction with ascorbic acid:** Due to the acidic nature of this diol, 1M PBS buffer was used to raise the pH to 7.4.

**Reaction with quercetin.** Owing to the very poor solubility of quercetin in a water environment a Tween 20 (a oligo(ethylene glycol) (polyoxyethylene) derivative of sorbitol monolaurate widely employed in bioassays and pharmaceutical formulations) was used. For quercetin concentrations up to 1 mM a 1:1 Tween/quercetin molar ratio was sufficient to completely solubilise it in 0.1 M PBS buffer, since its UV-Vis absorbance reaches a plateau and does not increase with larger amounts of surfactant. The highest concentration of Tween 20 used is limited by its ability to hamper the binding reaction. A maximum of 8% w/w of Tween 20 can be used without affecting the reaction kinetics.
Enzymatic Reactions. Typical experiments were performed in 96 well plates at 37 °C sealed with the help of PCR plate adhesive seals to prevent evaporation; three repetitions were carried out per sample in 0.1M PBS buffer.

- Demethylation of 3-Methoxytyramine. 3-methoxytyramine can bind to boronic acid only upon removal of its methoxy group, which can be carried out by cytochrome P450 variant 2D6. A stock solution of CYP2D6 was prepared by diluting 20 μL of a 500 pmol/mL solution with 520 μL of 0.1 M PBS. To this CYP solution, 8.34mg of NADPH was added which is needed for activation of the enzyme. The reaction of ARS with APBA was conducted as described above. Appropriate amounts of 3-methoxytyramine, ranging from a APBA : 3-methoxytyramine molar ratio of 1:0.5 to 1:3.2, were then added to each well, followed by 27 μL of the CYP2D6 stock solution (corresponding to 0.5 pmoles of CYP2D6 + 2mM final concentration of NADPH).

- Hydroxylation of estradiol. Estradiol can bind to boronic acid only upon the introduction of a second alcoholic function, which can be carried out by cytochrome P450 variant 1A2. Estradiol is barely soluble in the water medium that we have used for the diol/boronic reaction (0.1 M PBS) and thus requires a solubilising agent; since most surfactants would impair the activity of cytochrome P450, we have employed β-cyclodextrin sulphobutylether, (i.e an anionic, very water soluble cyclodextrin: neutral cyclodextrins did not provide sufficient solubilisation, while most surfactants do) in 1:1 molar ratio with estradiol. A stock solution of CYP1A2 was prepared by diluting 30 μL of a 1000 pmol/mL solution with 375 μL of 0.1 M PBS. To this CYP solution, 7.8mg of NADPH was added for its activation. 48 μL of ARS solution (final concentration: 0.56 mM) was added to each well, followed by 55 μL of APBA (final concentration: 0.56 mM); the mixture was allowed to react for 30 seconds (stable change in colour from red to orange). On completion of this reaction, a calculated amount of estradiol (APBA : estradiol molar ratios ranging from 1:0.1 to 1:4.5) was added to each well, followed by 27 μL of the CYP1A2 stock solution (corresponding to 2 pmoles of CYP1A2 + 2.5mM final concentration of NADPH).
Relevance of NADPH. NADPH (Scheme 2-3) is a coenzyme responsible for transferring electrons to cytochrome P450 for carrying out its various metabolic activities such as hydroxylation and demethylation\textsuperscript{69, 70}. CYP450 needs NADPH in order to act on its substrate since without it, its activity is seriously reduced (see appendix, Figure 2-10). In the abovementioned enzymatic reactions, both estradiol and 3-methoxytyramine do not possess a 1,2-diol and hence cannot link to any boronic acid moiety. The CYP1A2 and CYP2D6 using NADPH, help by hydroxylating\textsuperscript{71} and demethylating their respective substrates, thereby producing their respective 1,2-diols which can then link to APBA.

Control experiments without NADPH with all other parameters unchanged were carried out initially. Also control experiments without CYP450 and without NADPH were also carried out which showed no shift in wavelength due to the absence of any 1,2-diol.

Scheme 2-3. Structure of NADPH.
2.3.4 Analytical methods used for the calculation of binding constants

It is noteworthy that in this study we have not differentiated between the equilibria leading to the formation of the trigonal or the tetragonal form of the diol-boronic esters. Whenever binding constants are provided for the diol-boronic equilibria, they are overall affinities at pH=7.4 in 0.1 M PBS.

1) Determination of ARS/boronic acids binding constants through batochromic shifts

When using boronic acids such as APBA (here identified as B), that lack a significant absorption in the visible or near UV spectral region, the only absorbing species are ARS (here identified as A) and the ARS-boronic ester (here identified as AB), i.e. ARS in the free and bound form. As a result, the spectra of ARS with different amounts of boronic acid show a clear isosbestic point, ensuring the presence of only two species in the equilibrium (Figure 2-1, left). Since the spectra of ARS alone and of its complex, obtained from ARS in the presence of a large excess of APBA, can be accurately fitted with Gaussian equations (see appendix, Figure 2-6), it is possible to recreate the absorption spectrum of any mixture of ARS with its boronic ester, expressing it as a linear combination of the spectra of the two pure substances (see appendix, Figure 2-7):

$$A = x_A C_A e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e_1 e

where the absorbance A depends on the molar concentrations of the two products [expressed as the product of their molar fractions (xA – molar fraction of ARS, 1 - xA – molar fraction of ARS-APBA) times the total concentration of ARS species (CA)], and on parameters obtained from the spectra of the two pure products, $\lambda_{\text{max} 1}$ and $\lambda_{\text{max} 2}$, $e_1$ and $e_2$, $\omega_1$ and $\omega_2$, which are, respectively, the wavelengths of the maxima of the spectra of ARS and of its boronic ester, the corresponding extinction coefficients and parameters related to the band width.

By plotting the wavelength of the absorbance maxima of these calculated spectra vs. $x_A$ one obtains a sigmoidal graph (Figure 2-1, right); this graph is, in essence, a master curve, which allows to relate the location of the absorption maximum for a given
ARS/APBA mixture to the fraction of free ARS in that mixture. It is worth pointing out that this master curve is necessary, due to the non-linear nature of the relationship between the $\lambda_{\text{max}}$ of the solution and $x_A$.

\begin{align}
K_1 &= \frac{[AB]}{[A][B]} = \frac{[AB]}{(C_A-[AB])(C_B-[AB])} \\
[AB] &= \frac{C_A + C_B + \frac{1}{K_1}}{2} = \frac{(C_A + C_B + \frac{1}{K_1})^2 - 4C_AC_B}{2} \\
x_A &= 1 - \frac{[AB]}{C_A} = 1 - \frac{C_A + C_B + \frac{1}{K_1} - \sqrt{(C_A + C_B + \frac{1}{K_1})^2 - 4C_AC_B}}{2C_A}
\end{align}

It is then possible to calculate $K_1$ by applying a nonlinear least square regression to a set of experimental values $\lambda_{\text{max},i}$ from mixtures prepared at different initial concentrations of ARS and APBA ($C_{A,i}$ and $C_{B,i}$, respectively). This is obtained by taking into account

\begin{figure}
\centering
\includegraphics[width=\textwidth]{ARS-APBA_spectra.pdf}
\caption{Left: UV-Vis spectra of 0.56 mM ARS in the presence of increasing content of APBA. The isosbestic point is clearly visible at 497 nm. Right: the $\lambda_{\text{max}}$ of the curves obtained as linear combinations of the spectra of ARS and of the ARS/APBA complex show a sigmoidal dependence on $x_A$; by fitting it with a Boltzmann equation, it is possible to directly relate the experimental $\lambda_{\text{max}}$ of a mixture to the molar fraction of free ARS.}
\end{figure}
that the vector \((x_{A1}, \ldots, x_{An})\) can be expressed as function of \(K_i\), \(C_{Ai}\) and \(C_{Bi}\) (equation 4) and minimizing the sum of the squares of the offsets \(S = \sum_n(\lambda_{\text{max}}(x_{Ai}) - \tilde{\lambda}_{\text{max}}(x_{Ai}))^2\). Using a fixed concentration \(C_{Ai} = C_A = 5.6 \times 10^{-4}\) M and varying \(C_{Bi}\) in a range between \(5.6 \times 10^{-5}\) M and \(5.6 \times 10^{-3}\) M, the nonlinear least square regression (performed using a MATLAB-written routine) returned a value of the equilibrium constant \(K_i = 5150\) M\(^{-1}\) with a coefficient of determination \(R^2 = 0.9937\). An example of the convergence between the master curve \(\lambda_{\text{max}}(x_A)\) and the set of \((\tilde{\lambda}_{\text{max}}, x_{Ai})\) points \((x_{Ai} \) obtained from the optimization of \(K_i)\) is presented in appendix, Figure 2-8.

2) Determination of ARS/boronic acids binding constants through fluorescence

The binding of ARS to phenylboronic acid (PBA) and 3-aminophenlboronic acid (APBA) was also studied using the emission of the ARS boronic esters, recording the increase in fluorescence intensity of an ARS solution upon addition of the boronic acids, a method pioneered by Wang\(^72\). In those reports, fluorescence data were fitted according to

\[
\frac{1}{\Delta I_f} = \left(\Delta kp_0 I_0 K_i\right)^{-1} \frac{1}{[L]} + \left(\Delta kp_0 I_0\right)^{-1}
\]

(5)

where \(I_0\) is the total concentration of ARS (in our model it is called \(C_A\)), and \([L]\) should stand for the concentration of free boronic acid (in our model \([Bi]\)). Although this is not explicitly described in the paper, we are induced to believe that the total concentration of boronic acid \((C_B)\) was used instead of \([Bi]\): indeed by replacing \(C_B\) with \([Bi]\) our fluorescence data for the ARS/PBA equilibrium provide a constant substantially identical to that given by Springsteen et al.\(^72\) (see Table 2-1). We have, on the contrary used the expression of \([AB]\) provided by equation 3 to fit the fluorescence data through the model provided by equation 6

\[
FI = A^* \frac{C_A + C_B + \frac{1}{K_1} - \sqrt{(C_A + C_B + \frac{1}{K_1})^2 - 4C_AC_B}}{2}
\]

(6)
Where the fluorescence intensity $FI$ dependence on $C_B$ is used to calculate the unknown parameters $A$ and $K_1$ ($C_A$ being constant).

3) Determination of diol/boronic acid binding constants through competitive binding with ARS

The gradual addition of a diol (identified as $D$) to an ARS boronic ester determines an increasing batochromic shift due to the production of free ARS (Figure 2-2, left; see appendix, Figure 2-9, for a picture of the typical colour changes during the test).

![Figure 2-2](image)

**Figure 2-2.** Left: Shifts in $\lambda_{\text{max}}$ produced by the addition of increasing amounts of pyrogallol to a solution of ARS/APBA (both 0.56 mM). Right: Using equations 7 and 8 it is possible calculate the values of $K$ for any experimental point of the curve on the right, although we have generally limited this calculation to the points of the initial linear region of the curve. The average of these values is then used to calculate $K_2$, i.e. in this case the binding constant of pyrogallol to APBA.

The corresponding competitive equilibrium $AB + D \leftrightarrow DB + A$ is governed by a constant $K$ which can be expressed as the ratio of the two equilibrium constants for the formation of the individual boronic esters, $AB$ and $DB$ (equation 7), but can also be expressed as a function of the concentration of free ARS as the only variable (equation 8).

$$ K = \frac{[A][DB]}{[AB][D]} = \frac{[A][B][DB]}{[AB][B][D]} = \frac{K_2}{K_1} \quad (7) $$
\[
K = \frac{[A][DB]}{[AB][D]} = \frac{[A](C_B - [AB])}{(C_A - [A])[D]} = \frac{[A](C_B - C_A + [A])}{(C_A - [A])(C_D - C_B + (C_A - [A])(\frac{1}{[A]K_1} + 1))}
\] (8)

For each given amount of diol, one obtains a \( \lambda_{\text{max}} \) value from which it is then possible to calculate the fraction of free ARS \( x_A \), as shown in the previous section; the application of equation 8 allows then to obtain \( K \) and finally, through equation 7, the equilibrium constant of interest \( K_2 \). The values of \( K_2 \) were averaged through a range of concentrations (Figure 2-2, right).

4) Enzymatic reactions

In general, all enzymatic tests were based on the \textit{in situ} production of a diol in the presence of the ARS/APBA complex, taking part to a competitive equilibrium of the kind described in the previous section. Correspondingly, the main UV-Vis band of the solution undergoes a time- and concentration-dependent batochromic shift (Figure 2-3, left); the dependence on time provides information about the kinetics of the enzymatic conversion and that on the concentration of the precursor information about the binding constant of the diol.

\[\text{Figure 2-3. Left: example of the dependence of the batochromic shifts on time and concentration of the enzyme substrate for the enzymatic conversion of 3-methoxytyramine by CYP2D6 in the presence of the ARS/APBA complex (0.56 mM). Right: time dependence of the 3-methoxytyramineconcentration [S] ([S]_0 = 0.84 mM, [ARS/APBA] = 0.56 mM), compared to its fit with a simple exponential decay.}\]

In this part of the study we have always assumed that the enzymatic conversion is the rate-determining step of the test, i.e. the competitive equilibrium is established in an
instantaneous fashion as soon as new diol is produced. This assumption is reasonable, since the diol-boronic equilibria are established in a matter of seconds, while tenths of minutes are required to obtain quantitative yields in diols. We have also assumed that complete substrate conversion was obtained when the batochromic shifts reached their asymptotic values; in this way it is possible to replace $C_D$ in equation 8 with the initial substrate concentration $[S]_0$, therefore allowing the calculation of the diol-boronic equilibrium constant $K_2$.

It is then possible (see appendix, Additional information about enzymatic reaction experiments) to use $K_2$ to express the substrate concentration ($[S]$) as a function of the molar fraction of free ARS ($x_A$), which can be calculated from the batochromic shift; from the time dependence of $[S]$ it is possible to calculate the initial rate of the enzymatic reaction. In order to reduce the experimental error, this was done by fitting the time-dependent data as an exponential decay ($[S] = [S]_0 \exp(-kt)$, where the slope at $t=0$ is approximated to be $-k$); this approximation is rather coarse, but legitimate. The initial reaction rate $v$ was then plotted vs. $[S]_0$ and fitted using a Michaelis-Menten relationship $v = \frac{v_{max}[S]_0}{K_M + [S]_0}$.
2.4 Results and Discussion

2.4.1 Comparison absorbance vs. fluorescence assays

The UV-Vis spectrum of ARS is heavily affected by complexation: the absorption maximum of ARS in water is located at 519 nm (red colour), but it shifts to 480 nm (orange colour; see appendix, Figure 2-9) in the presence of a stoichiometric excess of boronic acids; this finding has been extensively reported in the literature. Analogously to what happens for the fluorescence intensity, also these batochromic shifts can be used to monitor the binding equilibrium; a detailed description of the method is provided in the experimental section.

We have compared the use of spectral shifts with that of fluorescence intensity the reactions of ARS with phenylboronic acid (PBA) and 3-amino phenylboronic acid (APBA) (Figure 2-4). An expected asymptotic behaviour was recorded for both assays with excess boronic acid; however, above all for ARS/APBA, the batochromic shifts provided better defined plots, possibly because of the fluorescence emission to aggregation/scattering at higher APBA concentrations.

![Figure 2-4](image.png)

Figure 2-4. Comparison of the variations in fluorescence intensity (right axes, data corrected for dilution) and location of the absorbance maximum (left axes) for a 0.56 mM ARS solution in 100 mM PBS as a function of the amount of added boronic acid. Please realize that the $\lambda_{\text{max}}$ data are related to the concentrations of the two chromophores (ARS and ARS boronic esters) in a non-linear fashion. Left: addition of phenyl boronic acid (PBA). Right: addition of 3-aminophenyl boronic acid (APBA). The $\lambda_{\text{max}}$ data appear to depend less than fluorescence ones on the nature of the boronic acid (possibly the introduction of the amino group in APBA provides a different quantum yield) and in the case of APBA show a better defined concentration dependence.
Although the equilibrium constants calculated with the two methods showed a statistically significant difference, their values were reasonably close; most importantly, the ARS/APBA $K_1$ was about 1.5-fold larger than that of ARS/APBA for both methods. We therefore concluded that the two methods were well comparable.

It is worth pointing out that the equilibrium constant for ARS/PBA calculated on the basis of the fluorescence measurements showed a significant difference from the literature value. This may be due to a certain variability caused by aggregation of the boronic acid at high concentration, but we cannot rule out a difference in calculation, as discussed in the Experimental Part, section “Determination of ARS/boronic acids binding constants through fluorescence” (Analytical methods, point 2). This is evident from Figure 2-4, Right which shows that the absorbance maximum reaches a plateau at a low concentration of APBA, while the fluorescence intensity keeps rising almost linearly in the concentration range tested.

<table>
<thead>
<tr>
<th>System</th>
<th>$K_1$ (M$^{-1}$)</th>
<th>Fluorescence</th>
<th>(Fluoresc. lit. method$^a$)</th>
<th>Batochromic shifts</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARS + PBA</td>
<td>2430 ± 110</td>
<td>(1245 ± 30; 1300$^{72}$)</td>
<td>3600 ± 150</td>
<td></td>
</tr>
<tr>
<td>ARS + APBA</td>
<td>3652 ± 415</td>
<td>(1474 ± 280)</td>
<td>5150 ± 200</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ here we report the $K_1$ value from ref. 72 for the ARS/PBA system and those calculated by us replacing the free boronic acid concentration with its total concentration, as described in the experimental section (Analytical methods, point 2).

### 2.4.2 Overall affinities ($K_2$) of different diols for APBA

As a model for possibly functional boronic acid, we have further investigated the binding strength of APBA with the compounds listed in Scheme 2-2 (Table 2-2). Since APBA has been rarely employed in diol/boron complexation equilibria, this choice does not allow a perfect comparison with literature data; however, we can qualitatively compare our data with those available for PBA (Table 2-2, 3rd column).
<table>
<thead>
<tr>
<th>Diol</th>
<th>$K_2$ (M$^{-1}$)</th>
<th>Opt. pH$^a$</th>
<th>$K_2$ (PBA) (M$^{-1}$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>0</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Veratrylamine</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aromatic diols</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrocatechol</td>
<td>1490 ± 70</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>1555 ± 66</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>Dopamine from 3-methoxytyramine</td>
<td>1550 ± 73</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>Epinephrine</td>
<td>1445 ± 65</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1545 ± 66</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>2-hydroxy estradiol from estradiol$^c$</td>
<td>955 ± 38</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>Aromatic polyols</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrogallol$^d$</td>
<td>1796 ± 78</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>Quercetin$^e$</td>
<td>1240 ± 98</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>Sugar diols</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>5.85 ± 1.2</td>
<td>10.5</td>
<td>13$^{72}$</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.75 ± 0.4</td>
<td>10.6</td>
<td>4.6$^{72}$</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>6.4 ± 0.94</td>
<td>10.4</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ the optimal pH was calculated according to $pH_{optimal} = \left( pK_{boronic} + pK_{(diol)} \right) / 2$

$^b$ literature values of PBA shown for comparison

$^c$ in the presence of sulphonated β-cyclodextrin to solubilise the steroid. The end point of the enzymatic reaction (see later) was used to calculate the value of the equilibrium constant, assuming complete conversion of estradiol.

$^d$ in the presence of TCEP to avoid pyrogallol oxidation

$^e$ in the presence of Tween 20 to solubilise quercetin
First of all, it is noteworthy that the two negative controls did not show any binding to APBA. The absence of competitive equilibria with veratrylamine, i.e. dimethylated dopamine, and phloroglucinol, with hydroxyl groups all in meta positions, ensured that the isolated OH groups and primary amines did not interfere with the assay. All aromatic diols and polyols exhibited very similar binding affinities to APBA, and all were significantly lower than that of ARS, showing therefore a negligible influence of the nature, size or polarity of the residues present in para position. The overall lower binding strength of catechols in comparison to ARS can be explained on the basis of their pKa. The optimal pH of binding is generally calculated as the average of the pKa of the two reactants. ARS has a pKa of 4, APBA of 8.9, which brings the optimal pH of binding to 6.45. Catechols have pKa values in the region of 9.2, thus an optimal pH value for binding should be in the proximity of 9.05. It is therefore not surprising that at neutral pH the binding strength of APBA with these catechols is considerably lower than that of ARS.

Among the aromatic diols/polyols only 2-hydroxyestradiol showed a significantly lower K2 value; this can be ascribed to both to the steric hindrance of the sulphonated β-cyclodextrin/2-hydroxy estradiol complex and the electrostatic repulsion between the reaction partners: both ARS/APBA and the cyclodextrin are negatively charged. Since in the case of quercetin the use of a non-ionic solubiliser did not appear to dramatically depress the binding affinity of the catechol, we are inclined to ascribe the lower K2 of 2-hydroxyestradiol mostly to the electrostatic effect.

The low binding constants recorded for sugar diols, in accordance to literature data, can be explained on the basis of the lower pKa of these compounds, in addition to a less favourable conformation of the vicinal OH groups. Indeed, the relative ranking of these compounds reflect the fact that mannose presents a more accessible cis diol and ascorbic an even more easily accessible enediol group.

The batochromic shift method has therefore allowed a rather precise estimation of the binding constant for a wide range of diols; further, it was successfully applied also on a heterogeneous sample (quercetin solubilised with Tween 20). This helps us in proving the usability of the method in various systems, especially in heterogeneous systems, and in having a benchmark for evaluating the binding strengths of these substances/drugs with a polymeric carrier. The binding constants of 2-hydroxyestradiol and quercetin are both reasonable at physiological pH showing that they can be coupled with boronic acids with ease using low amounts and would be easy to separate at acidic pH.
2.4.3 Enzymatic Reactions

We have successfully implemented the batochromic shift method to follow the kinetic of enzymatic reactions involving catechols. The plots of the initial reaction rates vs. substrate concentration for both the demethylation of 3-methoxytyramine by CYP2D6 and the hydroxylation of estradiol by CYP1A2 showed rather accurate Michaelis-Menten-type kinetics (Figure 2-5), which provided $v_{\text{max}} = 49.4 \pm 3.2 \text{ nmol/(min×pmol of CYP2D6)}$ and $K_m = 281 \pm 64 \text{ μM}$ for the first reaction, $v_{\text{max}} = 65.2 \pm 18.7 \text{ nmol/(min×pmol of CYP1A2)}$ and $K_m = 3.8 \pm 1.7 \text{ mM}$ for the second one. The activity of both enzymes, i.e. CYP2D6 as a demethylase and CYP1A2 as a hydroxylase, are NADPH-dependent; indeed the batochromic shift method allowed to verify that the absence of NADPH profoundly altered the enzymatic kinetics (see appendix, Figure 2-10).

![Figure 2-5](image)

Figure 2-5. Michaelis-Menten plots for the two enzymatic reactions followed in this study. Left: demethylation of 3-methoxytyramine. Right: hydroxylation of estradiol.

From a qualitative point of view, the most significant result is the verification that dopamine can be produced through the CYP2D6-mediated demethylation of 3-methoxytyramine, which on its turn is produced by COMT during the deactivation of dopamine, proving therefore the possibly antagonistic action of the two enzymes.

In quantitative terms, we do not have a direct literature comparison for the enzymatic parameters obtained through the batochromic shift method for this reaction; however, our $v_{\text{max}}$ and above all $K_m$ data are broadly comparable to literature examples the CYP2D6-mediated demethylation of $O$-methylphenols (Table 2-3). It is worth focusing
more on \( K_m \) than on \( v_{\text{max}} \), since the values of the latter are heavily affected by the purity of the enzymes, which varies considerably from microsomal preparations to recombinantly expressed molecules. The rather low \( K_m \) value can be in part ascribed to molecular features: the presence of a vicinal OH group in the 3-methoxytyramine structure may decrease the affinity of the enzyme for the substrate; however, the sourcing of CYP2D6 is also likely to play a role. A more accurate comparison of the batochromic shift method with other reports would appear to be possible for the kinetic parameters of the enzymatic 2-hydroxylation of estradiol. For most literature reports, \( K_m \) values clustered in the proximity of 20 \( \mu \text{M} \) (Table 2–4). It must be pointed out these literature studies were performed with substrate concentrations as high as 200 \( \mu \text{M} \), while the solubility of estradiol in water or water buffers is lower than 20 \( \mu \text{M} \) \cite{76}; therefore, it is not unlikely that the reported data reflect the reactivity of an at least partially phase separated estradiol. In this study, on the contrary, we have used considerably higher estradiol concentrations, in the range 500–2500 \( \mu \text{M} \), where the steroid was kept in a molecularly dispersed state by the use of a sulphonated cyclodextrin. Normal cyclodextrin has 1,2-diols present which can link with boronate species, and hence on performing a preliminary experiment with APBA and cyclodextrin, complexation was noticed (data not shown). Therefore, we used \( \beta \)-cyclodextrin sulphobutylether, which has the advantage of being highly water soluble as well as not containing the 1,2-diol groups, and hence showed no linkage with the boronate species when the control experiment without estradiol and CYP1A2 was carried out. We have observed a dramatically lower affinity of CYP1A2 for the substrate, with \( K_m \) about two orders of magnitude higher than literature values. We attribute this major difference primarily to the effect of the high anionic charge and of the steric hindrance of estradiol/cyclodextrin complex, which surely detrimentaly affect binding. However, we cannot exclude literature reports to overestimate the affinity of CYP1A2 for estradiol: since the steroid was frequently used at concentrations considerably above its solubility in water, the affinity may be influenced by its aggregation and increased local concentration.
Table 2-3. Comparison of the kinetic parameters for the CYP2D6-mediated demethylation of some O-methylated phenols (literature data, see appendix, Figure 2-11, for the structures of the compounds) and of 3-methoxytyramine (this study).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_M ) (enzyme isoform)(^a) (µM)</th>
<th>( v_{max} ) (pmol/pmol P450/min)</th>
<th>Demethylase activity (CLint)(^b) (µL/pmol P450/min)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinoline ((\rightarrow6)-hydroxy-1,2,3,4-tetrahydro-(\beta)-carboline)</td>
<td>0.74 (CYPD26.1)</td>
<td>3.06 (CYPD26.1)</td>
<td>4.13</td>
<td>77</td>
</tr>
<tr>
<td>5-methoxytryptamine (5-MT) ((\rightarrow)serotonin)</td>
<td>2.3 (CYPD26.2)</td>
<td>1.75 (CYPD26.2)</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>26(^c)</td>
<td>15(^c)</td>
<td>78</td>
</tr>
<tr>
<td>5-methoxy-N,N-dimethyltryptamine (5-MDMT) ((\rightarrow)bufotenine)(^79)</td>
<td>29.3 (CYPD26.1)</td>
<td>12.3 (CYPD26.1)</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>85.0 (CYPD26.2)</td>
<td>14.4 (CYPD26.2)</td>
<td>0.17</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>947 (CYPD26.10)</td>
<td>10.3 (CYPD26.10)</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>Dextromethorphan ((\rightarrow)dextrorphan)</td>
<td>2.7 (high affinity)</td>
<td>2.8(^d) (high affinity)</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>757 (low affinity)</td>
<td>136(^d) (low affinity)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>10200(^d)</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>Omeprazole ((\rightarrow5)-O-desmethylomeprazole)</td>
<td>13.6 (high affinity)</td>
<td>=</td>
<td></td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>139 (low affinity)</td>
<td>=</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methoxytyramine</td>
<td>281(^e)</td>
<td>49.4</td>
<td>0.18</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\) in bracket the name of the specific isoform used (if specified) or the definition of the enzymatic form if the kinetic analysis showed bienzymatic activity.

\(^b\) CLint (intrinsic clearance = \( v_{max} / K_M \)) is a common measure of the enzymatic activity in the cell.

\(^c\) \( K_{cat} \) and \( \frac{K_{cat}}{K_M} \) values, respectively expressed in min\(^{-1}\) and min\(^{-1}\)/µM.
expressed in pmol/mg protein/hour for the conversion operated by rat cerebellar membranes with an unspecified concentration of CYP2D6.

Mixture of low-affinity recombinant human and yeast CYP2D6 (producer: SPI-Bio, Montigny-le-Bretonneux, France)

Table 2-4. Comparison of the kinetic parameters for the CYP1A2-mediated 2-hydroxylation of estradiol (literature data and this study) or similar steroidal compounds (literature data).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ (µM)</th>
<th>$v_{max}$ (pmol/pmol P450/min)</th>
<th>Hydrolase activity (CLint) (µL/pmol P450/min)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td>7.7</td>
<td>0.17</td>
<td>=</td>
<td>83</td>
</tr>
<tr>
<td>Ethinyl estradiol</td>
<td>19.0</td>
<td>9.2</td>
<td>0.48</td>
<td>84</td>
</tr>
<tr>
<td>Estradiol</td>
<td>73</td>
<td>1.30</td>
<td>0.018</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>27.5</td>
<td>17.4</td>
<td>0.65</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>20.6</td>
<td>11</td>
<td>0.53</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>~19</td>
<td>~7</td>
<td>0.3-0.4</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>52.1</td>
<td>1.02</td>
<td>0.019</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>3800</td>
<td>65</td>
<td>0.017</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.5 Conclusions

The use of ARS as an absorbance reporter allowed to accurately determine the equilibrium constants of a number of diol/boronic couples and the results were comparable to those obtained using fluorescence-based assays. The batochoromeric shift method, on the other hand, allowed to study also more concentrated and possibly heterogeneous samples, allowing for example to monitor this “click” reaction in a self-emulsifying system (Tween 20 + quercetin) and during enzymatic kinetics, also performed on solubilised active principles (cyclodextrin + estradiol), showing promise as a reporter for performing diol/boronic “click” reactions even in biological environments.

As a side result of this study, we have also for the first time demonstrated that CYP2D6 is able to demethylate the monomethyl ether of dopamine (3-methoxytyramine). One of the major drawbacks of Parkinson’s disease (PD) therapies based on the use of catecholamine drugs or pro-drugs (e.g. L-DOPA) is the inactivation of exogenous catecholamines through methylation (catechol O-methyltransferase, COMT) or deamination (monoamine oxidases, MAOs). The first pathway is countered using combinations of catecholamines with COMT inhibitors and it is complicated by the COMT functional Val158Met polymorphism\(^{65, 88, 89}\), whose incidence is so high that is also used as an index of dopamine bioavailability\(^{90}\). CYP2D6 is highly expressed in the brain\(^{91}\); there, it can produce dopamine via 2-hydroxylation of tyramine\(^{92}\), but it has also been shown to have a demethylating activity\(^{93}\). CYP2D6 has an extended polymorphism, which is well known e.g. to affect opioid efficacy\(^{94}\) (O-demethylation of codein to morphin). Several studies have also suggested a relationship between CYP2D6 polymorphism and incidence of PD, although there are also conflicting reports as reviewed by BenMoyal-Segal and Soreq\(^{95}\), and this possible effect has been mostly attributed to the catabolism of xenobiotics, e.g. pesticides.

Here we have shown that CYP2D6 is able to regenerate dopamine from its COMT-derived inactivation product, 3-methoxytyramine. This suggests that the efficacy of a catecholamine-based PD therapy may be influenced by the interplay between these two enzymes and by their polymorphisms; for example, high CYP2D6 metabolizers may require lower dosages of COMT inhibitors to achieve high dopamine concentrations in the brain.
2.6 Appendix

Figure 2-6. UV-Vis spectra of Alizarin Red S and its complex with APBA (10-fold excess of APBA) compared to the corresponding single-peak Gaussian fits. The experimental and fitted curves are almost perfectly superimposable throughout the investigated spectral range.

Figure 2-7. Calculated absorption spectra of ARS solutions with different molar fractions of APBA. The spectra were generated as a linear combination of the spectra presented in Figure 2-6.
Figure 2-8. Example of agreement between “master curve” and the calculated data points.

Figure 2-9. Typical competitive assay test run in a 96-well plate. For each experiment of diol/ARS/APBA equilibrium 24 wells were used for assessing different diol/ARS molar ratios. In the picture, the well plate shows three experiments (two rows each) and the two controls, i.e. ARS alone and its complex with APBA.
Figure 2-10. Comparison of Michaelis-Menten plots and of the corresponding kinetic parameters for the two enzymatic reactions performed in the presence and in the absence of NAPDH.

Figure 2-11. CYP2D6-mediated demethylation reactions considered in Table 3.
Additional information about enzymatic reaction experiments

Considering the contemporaneous presence of ARS ($A$), APBA ($B$), a diol precursors, which is also the enzyme substrate ($S$) and the diol ($D$), the overall mass balance of the enzymatic reactions coupled to the diol/boronic equilibria comprises the following equations:

\[
C_A = [A] + [AB]
\]

\[
C_B = [B] + [AB] + [DB]
\]

\[
C_D = [D] + [DB]
\]

\[
C_S = [D] + [DB] + [S]
\]

Therefore

\[
[S] = C_S - [D] - [DB]
\]

Since $[D] = \frac{[DB]}{[B]K_2}$, one obtains $[S] = C_S - \left(\frac{1}{[B]K_2} + 1\right) [DB]$

Since $[B] = \frac{[AB]}{K_{1[A]}}$ and $[DB] = C_B - [B] - [AB]$,

then $[S] = C_S - \left(\frac{[A]K_1}{[AB]K_2} + 1\right) \left( C_B - \frac{[AB]}{[A]K_1} - [AB] \right)$

Rearranging the expression, one obtains

\[
[S] = C_S - \left( C_B - [AB] \left( \frac{1}{[A]K_1} + 1 \right) \left( \frac{[A]K_1}{[AB]K_2} + 1 \right) \right) \text{ and,}
\]

since $[A] = x_A, C_A$ and $[AB] = (1 - x_A)C_A$, it is finally possible to express the concentration of the enzyme substrate as a function of the molar fraction of free ARS, which is measured through the batochromic shift method:

\[
[S] = C_S - \left( C_B - (1 - x_A)C_A \left( \frac{1}{x_A C_A K_1} + 1 \right) \right) \left( \frac{x_A K_1}{(1 - x_A)K_2} + 1 \right)
\]
2.7 References

68. Subrahmanyam, V.; Renwick, A. B.; Walters, D. G.; Young, P. J.; Price, R. J.; Tonelli, A. P.; Lake, B. G. Drug Metabolism and Disposition 2001, 29, (8), 1146-1155.
3. Bioconjugation of hyaluronic acid using boronic/diol coupling as a “click” reaction

3.1 Summary

This study focuses on the development of a bioconjugation strategy based on boronic/diol complexation, which was used as a sort of reversible “click” reaction to derivatise hyaluronic acid (HA) as a model biomolecule. Boronic- and catechol-functionalised HA derivatives were thus prepared and characterized in terms of cytotoxicity (on L929 fibroblasts and J774 macrophages) and enzymatic degradability. Due to a better cytotoxicity evaluation, boronic acid derivatives were chosen for further investigation, focusing on a derivative where 25% of repeating units were derivatised with boronic acid. The binding strength of this polymer with a library of low molecular weight or polymeric diols showed this macromolecular derivative to perform slightly better than a low molecular weight model compound (3-aminophenyl boronic acid, 3-APBA), with equilibrium constants generally averaging $2 \times 10^3$ M$^{-1}$ (Kd<500 µM) with catechols.
3.2 Introduction

We here present a bioconjugation approach based on the use of boronic acid/diol complexation as a reversible “click” reaction. The reaction between boronic acids and *cis* diols, in particular catechols, is quantitative and selective and orthogonal to most biochemical reaction pathways. Boronic acids have therefore been widely employed for the selective recognition of sugars and expressing a behaviour substantially analogous to lectins. Inter alia, this reaction has allowed also the decoration of the surface of living cells (their glyocalyx) with synthetic polymers. Through an appropriate choice of the diol reactand, e.g. a catechol, this reaction is characterized by low dissociation constants (< 1 mM (equilibrium constants > 10^3 M^-1)) at physiological pH, and it is reversible at acidic pH. Probably, the most popular experimental method for the determination of boronic/diol equilibrium constants is the use of competitive equilibria Alizarin Red S (ARS), which is an aromatic diol exhibiting large spectral changes upon binding to boronic derivatives: for example, ARS develops a strong fluorescence in the boron-bound form. In our previous studies, we have preferred to use the shifts of the absorbance maximum of ARS, which are less sensitive to scattering and also to the possible presence of quenchers, and therefore can be employed also in complex (biological) environments. This method has allowed to measure the equilibrium constants between 3-aminophenylboronic acid (3-APBA) and a number of diols in water solution or dispersion, and to follow the kinetics of enzymatic reactions producing catechol compounds, e.g. the O-demethylation of 3-methoxytyramine and the 2-hydroxylation of cyclodextrin-solubilised estradiol, which are operated by two members of the cytochrome P450 family, respectively CYP2D6 and CYP1A2.

In the present study we have focused on the use of the boronic/diol complexation as a bioconjugation reaction, using hyaluronic acid (HA) as a model polymeric substrate. Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan composed of alternating D-glucuronic acid and N-acetyl-D-glucosamine monomeric units. It is an ubiquitous component of virtually all extracellular matrices, although it is specifically abundant in the vitreous humour, vocal folds, synovial fluid, umbilical cord, dermis, subcutaneous tissue and cartilage where it contributes to viscosity, tissue flow, tissue osmosis, shock absorption, wound healing and space filling. Clinically, HA has been employed in a number of applications, ranging from synovial fluid replacement,
to the management of skin lesions, and including vaginal dryness, nasal dryness, gingival inflammatory conditions, lubrication of eye and eye surgery. Recently, HA is increasingly looked at as the main component of carrier structures for controlled and possibly targeted drug delivery; see for example the recent review of Ossipov for HA carriers in cancer. The advantages of HA as a carrier structure are numerous:

1) HA is a natural product which has an essential role in the rheology of several body fluids, and its human form is identical to that of any other mammal;

2) HA is non-inflammatory, at least in its high molecular weight form;

3) HA cellular uptake is mediated by its interaction with cell surface receptors generally known as hyaluronan binding proteins or hyaladherins, the most common of which are CD44 and RHAMM. CD44 is particularly interesting: this receptor is constitutively expressed in a large number of cells and is involved in a number of functions: cell-cell aggregation, retention of pericellular matrix, matrix-cell and cell-matrix signalling, receptor mediated degradation of hyaluronan, and cell migration. However, CD44 affinity to HA appears to be inflammation-dependent, possibly through the cleavage of sialic acid residues. This opens the possibility to utilize HA-based structures for a selective delivery of active principles to inflammation-activated cells.

4) HA is enzymatically degradable. Hyaluronidases are a group of rather unspecific enzymes, since they all degrade also chondroitin and chondroitin sulphate; all vertebrate hyaluronidases are hydrolysases (while bacterial ones are lyases) that degrade HA to low polymers and/or oligomers, which can be further degraded by other enzymes, e.g. β-D-glucuronidase and β-N-acetyl-D-hexosaminidase. In the human genome, six HYALs have been recognized: HYALs 1 to 4, HYALP1, and PH-20 which are distributed at various different sites in the body and are responsible for HA turnover. Having a large number of HYAL enzymes distributed throughout the body, ensures biodegradability of HA based carriers – the only limitation being that they are still recognizable by these enzymes.

In the present work we have synthesised derivatives of HA bearing catechols (from dopamine), dimethylated catechols (acting as a negative control, from veratrylamine)
and boronic acids (from 3-aminophenylboronic acid, 3-APBA) as functional groups; the functionalisation was accomplished using a well-established procedure\textsuperscript{31,32} based on the use of EDC ([3-(dimethylamino)propyl]ethylcarbodiimide) and sulfoNHS (N-hydroxy2-sulphosuccinimide) as promoters of the reaction between the carboxylic acid groups of HA and primary amines (Scheme 3-1).

\textbf{Scheme 3-1.} Synthetic process and structures of HA derivatives and their complexes.

We have then tackled the issue of which group (boronic or catechol) would better allow a better conjugate performance (biocompatibility and degradability) for a given HA structure. The functional HA derivatives were then used in boronic/diol equilibria. Specifically, we have focused on boronic acid-containing HA due to its more favourable toxicity profile (see results and discussion) and studied its complexation with a number of diols: sugar diols, high molecular weight catechols (HA-dopamine and PEG-dopamine) and low molecular weight catechols, some \textit{in situ} enzymatically generated (dopamine, 2-hydroxyestradiol) and some dispersed in water with the help of solubilisers (quercetin, 2-hydroxyestradiol). As a comparison, the complexation of HA-dopamine with 3-APBA was studied too. A complete list of the compounds used in these equilibria is provided in Scheme 3-2.
Scheme 3-2. Structures of diols (columns in the left and in the centre) and boronic acids (right column) used in this study. The binding constants of 3-APBA with all low molecular weight diols were already measured in a previous study.8
3.3 Experimental Section

3.3.1 Materials

High molecular weight hyaluronic acid sodium salt (HA) \([\text{MW} = 1.10 \times 10^6 \text{ g/mol}; \overline{M}_w = 1.02\pm0.22 \times 10^6 \text{ g/mol and } R_g = 111\pm10 \text{ nm (static light scattering)}]\) was purchased from Medipol SA, (Lausanne, Switzerland). Hydrochloric acid (32\%) (HCl), \(N\)-(3-dimethylaminopropyl)-\(N'\)-ethylcarbodiimide (EDC), \(N\)-hydroxysulphosuccinimide sodium salt (Sulpho-NHS), L-ascorbic acid, dopamine hydrochloride (DOPA), 3-aminophenylboronic acid hydrochloride (3-APBA) and veratrylamine were supplied as high purity reagents (purity always \(\geq 98\%\)) by Sigma-Aldrich (U.K.) and used without further purification. Alizarin red S (ARS), D- (+)-mannose, D- (+)-glucose, pyrogallol, phloroglucinol, quercetin, tween 20, estradiol, pyrocatechol, (±)-epinephrine hydrochloride, DL-norepinephrine, Cytochrome P450 human 1A2, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and sodium hydroxide pellets (NaOH) were supplied as high purity reagents (purity always \(\geq 98\%\)) by Sigma-Aldrich (U.K.) and used without further purification. Monomethoxy poly(ethylene glycol), \(M_n=2,000 \text{ g/mol, functionalised with dopamine (PEG-DOPA) was synthesised by Dr. T. Kotsokechagia in a two-step reaction sequence (reaction of MeO-PEG-OH with } p\text{-nitrophenyl chloroformiate and then reaction of the resulting } MeO-PEG-(p\text{-nitrophenyl)carbonate with dopamine) and kindly provided for this study. Ovine testicular hyaluronidase (HAse, activity=9502 units/mg) was purchased from Calbiochem (Darmstadt, Germany). Human Cytochrome P450 2D6 Yeast reductase was supplied by Immuno diagnostic systems (U.K.). \(\beta\)-cyclodextrin sulphobutylether (Captisol) was supplied by Cydex Pharmaceuticals (U.S.). All solutions for binding studies were prepared in 100 mM PBS obtained by dissolving sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate dihydrate and sodium chloride, supplied by BDH (U.K.), in concentrations respectively of 2.3 g/L, 11.8 g/L and 9 g/L in water purified in a Milli-Q system (Millipore, U.K.).
3.3.2 Physico-chemical characterization

**UV spectroscopy.** A BioTek Synergy 2 multi-mode microplate reader was employed to record absorbance spectra. The spectra of enzymatic reactions were recorded in the range 450-550 nm and at 37 °C, and were corrected by subtracting the possible scattering component of the buffer solution. All other spectra were recorded at 25 °C. The molar percentage of functionalised units were calculated on the basis of the previously measured values of molar extinction coefficient of dopamine $\varepsilon_{280} = 2610$ L mol$^{-1}$ cm$^{-1}$, veratrylamine $\varepsilon_{277} = 2739$ L mol$^{-1}$ cm$^{-1}$ and 3-APBA $\varepsilon_{296} = 327$ L mol$^{-1}$ cm$^{-1}$; The absorbance at the respective wavelengths could be related to the weight fraction of functionalised units ($W$) and, through the molecular weights of the functionalised monomeric units ($MW$) and of the non-functionalised monomeric units ($MW_{HA}$) it is then possible to calculate the molar fraction of functionalised units ($x$), using the following equation:

$$A_{280} = \varepsilon_{280} L \frac{C_{HA} W}{MW} = \varepsilon_{280} L C_{HA} x \frac{1}{MW + (1-x)MW_{HA}}$$

**$^1H$ NMR spectroscopy.** Spectra were recorded on 0.5 wt.% polymer solutions in deuterated water using a 300 MHz Bruker spectrometer. Once the spectrum for the $^1$H-NMR was obtained, the percentage of derivatisation was calculated by integrating the area below the peaks for the amine (e.g. dopamine) and the acetyl peak for HA and comparing these integrated values using the following equation:

$$\frac{\text{Integral of reference peak}}{\text{Number of } H \text{protons of reference peak}} = \frac{\text{Integral of sample peak}}{\text{Number of protons of sample peak}} \times \frac{\text{Number of protons modified}}{\text{Number of protons modified}}$$

Once the number of protons modified were obtained, the degree of derivatisation was calculated using the following equation:

$$\text{Derivatisation Percentage} = \frac{\text{Number of protons modified}}{100}$$

**Viscometry.** The viscosity of HA solutions was measured with a falling-ball AMVn microviscometer (Anton-Paar, Hertford, UK) operating at 37°C and employing a capillary of 1.6 mm internal diameter used at an angle of 70°.
**Static light scattering (SLS).** 2 mL of 3, 2.2, 1.5, 0.8 and 0.1 mg/mL solutions of HA or HA derivatives in 0.1 M PBS were filtered 10 times through 0.22 µm hydrophilic PES filters and the absence of dust was confirmed by dynamic light scattering. SLS measurements were carried out at a constant temperature of 20°C with a Melles Griot HeNe class 3B laser (75 mW maximum power at 632.8 nm) coupled with a Brookhaven instruments goniometer and photodiode cascade detector. Measurements were performed at scattering angles from 30 to 140° with a 10° stepwise increase. Toluene was used as a calibration standard. The weight-average molecular weight (\(M_w\)), Z-average radius of gyration (\(R_g\)), and second virial coefficient (\(A_2\)) values were estimated from the relation \(H_c = \frac{1}{M_w} \cdot \left[ \frac{1}{3} + \frac{\Delta R}{K_I c} \right] + 2A_2 c\) where \(\Delta R\) is the Rayleigh ratio and it is assumed that \(\Delta R = K_I c\), \(K = 4\pi n^2 (dn/dc)^2 / N_A \lambda^4\), \(n\) the refractive index of the solvent, \(dn/dc\) the refractive index dependence on the solute concentration, and \(N_A\) is Avogadro’s number. \(dn/dc\) was 0.1518 ± 0.015 mL/g for HA, 0.1526 ± 0.01 mL/g for HA-BA and HA-BA:DOPA, 0.1579 ± 0.005 mL/g for HA-veratrylamine, 0.0885 ± 0.012 mL/g for HA-DOPA and 0.1009 ± 0.011 mL/g for HA-DOPA:BA (see appendix, Table 3-5) as determined using a Bellingham and Stanley refractometer with a sodium lamp at 589.6 nm. By measuring the scattering intensity for a set of \(\theta\) and \(c\) and correspondingly producing Zimm plots (see appendix, Figure 3-5), the values of \(M_w\), \(R_g\), and \(A_2\) were estimated.

**\(pK_a\) determination.** The \(pK_a\) of HA-3-APBA (X2) was determined by titrating 2 mL of a 4.91 mg/mL solution of X2 (0.003 M boronic groups) with 10 mM NaOH solution in a 10 mL glass vial. 100 µL of 10 mM NaOH was added every minute, and the pH was recorded every 30 seconds. The solution was added until the point that the \(pH\) became stable and would not change any further on addition of further NaOH. The \(pK_a\) was estimated to be 8.4 by plotting a graph of amount of NaOH added versus \(pH\) (see appendix, Figure 3-9) and finding the half equivalence point that is where half the amount of titrant has been added to neutralize the acid.
### 3.3.3 Synthesis of HA derivatives

The conditions for the synthesis of dopamine-containing HA derivatives were optimized varying the molar ratios between different reactants (carboxy groups, EDC, sulpho-NHS and amines) and the ratios conducing to the highest yields (Table 3-1) were then employed for the preparation of veratrylamine and 3-APBA derivatives.

In a typical experiment, a Radleys 6 chamber parallel reactor (each chamber – 50 mL) was used in which in each chamber the HA concentration was fixed (24 mL of a 6.15 mg/mL stock solution of HA used, 7.34 mM COO⁻ groups) and the required concentrations of EDC, sulpho-NHS and amines were added in various chambers depending on the molar ratio needed.

As an example, 570 µl of a 50 mg/mL EDC solution in deionized water (freshly prepared) were added to 24 mL of a 6.15 mg/mL HA solution (7.34 mM COO⁻ groups, 3.68 mM EDC, for a 1:0.5 COO⁻ to EDC molar ratio). The mixture was allowed to react for 30 seconds under continuous stirring prior to the addition of 1.66 mL of a 24 mg/mL Sulpho-NHS solution in deionized water (3.68 mM sulpho-NHS, for a 1:1 sulpho-NHS/EDC molar ratio). After 1 minute, variable volumes of a freshly prepared 63 mg/mL solution of amine (dopamine, veratrylamine or 3-APBA) hydrochloride were finally added to different chambers to achieve the desired COO⁻ of HA : EDC : amine molar ratio; in case of dopamine the amine solution contained ascorbic acid in a 1:1.5 dopamine/ascorbic acid molar ratio. The mixture was allowed to react overnight under stirring at room temperature. The samples were then purified using ultrafiltration using membranes with MWCO = 10,000 g/mol and freeze dried. Average yields (weight of recovered material/weight of recoverable material) = 80 - 90% wt. A detailed list of the conversion of carboxy groups as a function of the molar ratios between reactants is provided Table 3-1.

**UV (deionized water):** HA-Dopamine: 280 nm. HA-Veratrylamine: 277 nm. HA-3-APBA: 296 nm.

**¹H-NMR (D₂O):** HA-Dopamine: δ = 6.58 – 6.63 (H-dopamine), 6.68 – 6.71 (H-dopamine), 6.73 – 6.78 (H-dopamine), 4.8 (H-1 of GlcNAc), 4.3 – 4.5 (H-1 of GlcA), 3.1 – 3.8 (H-2, H-3, H-4, H-5, H-6a, H-6b of GlcNAc, H-2, H-3, H-4, H-5 of GlcA), 3.05 – 3.15 (α (benzylic) CH₂ dopamine), 2.7 – 2.82 (β CH₂ dopamine), 1.8 – 1.9 ppm (Acetyl H-8 of GlcNAc). HA-Veratrylamine: δ = 6.9 - 7 (3×H veratrylamine), 4.8 (H-

**HA-3-APBA:** δ = 7.36 – 7.42 (H APBA), δ = 7.49 – 7.6 (2\times H APBA), 7.76– 7.81 (H APBA), 4.8 (H-1 of GlcNAc), 4.3 – 4.5 (H-1 of GlcA), 3.1 – 3.8 (H-2, H-3, H-4, H-5, H-6a, H-6b of GlcNAc, H-2, H-3, H-4, H-5 of GlcA), 1.8 – 1.9 ppm (Acetyl H-8 of GlcNAc).

### 3.3.4 Enzymatic degradability of HA derivatives

The concentration of the polymers was adjusted to provide an initial dynamic viscosity of about 20 mPa\times sec; this corresponded to concentrations of 16 mg/mL for P1, 18 mg/mL for P2, 3 mg/mL for T1, 16 mg/mL for T2, 5 mg/mL for X1, 9 mg/mL for X2 and 1 mg/ml for the precursor HA. A 0.01 mg/mL enzyme stock solution was prepared in deionized water (95.02 units/mL) and polymers were tested in solution form at 37°C. It is noteworthy that L-ascorbic acid was not added to P1 or P2 solutions in order to prevent any interference of the reducing agent with the degradation. An exponential decay model \( \eta_{\text{rel}}(t) = \eta_{\text{rel}}(\infty) + (\eta_{\text{rel}}(0) - \eta_{\text{rel}}(\infty))e^{-t/\tau} \) was used to fit the time dependence of dynamic viscosity. The parameter \( 1/\tau \) is related to the reaction rate of enzymatic hydrolysis; however, direct comparison between different samples is not possible, due to their different concentrations, which provide a different concentration of degradable units. We have therefore calculated a “normalized reactivity” related to the reaction rate per enzyme-binding (negatively charged) disaccharidic unit, by dividing \( 1/\tau \) by the molar concentration of non-derivatised units.
Table 3-1. Conversion of carboxylic groups to amides as a function of the molar ratios between reactands. The underlined conditions were selected for further characterization studies, those in bold for the studies of diol/boronic binding.

<table>
<thead>
<tr>
<th>Amine</th>
<th>Molar Ratio</th>
<th>Degree of Derivatisation (mol %)</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>UV</td>
<td>NMR</td>
</tr>
<tr>
<td>Dopamine</td>
<td>1:0.5:0.5:0.5</td>
<td>8.4</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>1:2:2:2</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1:2:2:4</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>1:2:2:6</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1:2:4:4</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1:2:4:6</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1:4:4:4</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1:4:4:6</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1:4:6:6</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1:4:6:8</td>
<td>11</td>
<td>12</td>
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<td></td>
<td>1:6:6:6</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1:6:6:8</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Veratrylamine</td>
<td>1:0.5:0.5:0.5</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>1:2:2:4</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>3-APBA</td>
<td>1:0.5:0.5:0.5</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1:2:2:4</td>
<td>24</td>
<td>25</td>
</tr>
</tbody>
</table>

* No ascorbic acid, due to the absence of oxidisable groups.

3.3.5 Evaluation of cytotoxicity of HA derivatives on fibroblasts and macrophages

In metabolically active cells, dehydrogenase enzymes produce NADPH or NADH that can reduce MTS (a tetrazolium compound) to formazan. By measuring the optical density (OD) of the latter, the cytotoxicity of a derivative in solution can be measured as the percentage of \([\text{OD}_{\text{material}} - \text{OD}_{\text{blank}}]/\text{OD}_{\text{control}}\). Murine L929 fibroblasts and J774.2 macrophages (ECACC, UK) were maintained as, respectively, adherent and semi-adherent cell culture at 37°C in humidified atmosphere (5% CO2) in Dulbecco modified Eagle’s minimal essential medium (DMEM, 25 mM glucose) supplemented with 2 mM
glutamine (Gibco), 10% heat inactivated fetal calf serum (FCS) (Invitrogen, UK), 100 IU/ml penicillin and 100 IU/ml streptomycin (Gibco). For L929 fibroblasts cells splitting, trypsin-EDTA (Invitrogen, UK) consisting of 2.5% w/v of trypsin and 0.2% w/v EDTA in PBS was used, while J774.2 macrophages cells were detached by scraping. Both cell lines were adjusted to the required concentration of viable cells, by counting in a haemocytometer in the presence of 0.4% trypan blue. Upon confluence (~80%), cells were seeded on 96-well plates at a concentration of 4x10^4 ml^-1 and left to adhere for 24 hours in a humified incubator at 37°C and 5% CO2. The following day, solutions of the HA derivatives [HA-Dopamine (P1=8.5%, P2=16%), HA-Veratrylamine (T1=16%, T2=27%) and HA-3-APBA (X1=17%, X2=25%)] in full DMEM at variable concentration (10, 7.5, 5, 1, 0.5, 0.1 and 0.01 mg/mL) were added and left to incubate for 24 hours. The HA-Dopamine DMEM solutions contained also L-ascorbic acid (0.55 mg/mL and 1.03 mg/mL, respectively for P1 and P2) to prevent the oxidation of catechol groups. All solutions were then removed; the wells were washed with PBS, then serum-free DMEM including 4.8% CellTitre 96® Aqueous One Solution (Promega, Southampton, UK), and were left to incubate for 3 hours. The amount of soluble formazan produced by mitochondria of viable cells was then measured by recording the absorbance at 490 nm. Eight wells were exposed to each treatment (n=8) and the experiment was repeated three times.

3.3.6 Evaluation of boronic/diol equilibrium constants

**Batochromic shift method:** the mathematical model for the calculation of boronic/diol equilibrium constants from the batochromic shifts of ARS (corresponding to the liberation of free ARS from an ARS/boronic complex) was described in Chapter 2, pages 88 - 93.

**Experimental conditions:** For competitive binding experiments in each well of a 96 well plate (total volume of each sample = 250 µL) 48 µL of a 1 mg/mL ARS solution and 55 µL of a 3.72 mg/mL HA-3-APBA (X2) solution (both in 0.1 M PBS) were mixed to obtain a 0.56 mM final concentration for both reagents (ARS and boronic in HA-3-APBA) and were allowed to react for 30 seconds; the colour of the solution correspondingly changed from red to orange. An amount of diol corresponding to HA-
3-APBA/diol molar ratios ranging from 1:0.5 to 1:1000 was then added, producing a clear orange to red chromatic change at high HA-3-ABPA/diol molar ratios. The highest HA-3-APBA:diol molar ratio for veratrylamine and for phloroglucinol was 1:10 (0.0056 M) and 1:100 (0.056 M) respectively, due to their limited solubility in water. All the absorbance values and the concentrations of the reagents were then corrected for the dilution. In simple binding experiments the same protocol was used, but the volumes of HA-3-APBA solutions were varied in a series of experiments and the absorbance values were monitored to obtain the intensities as a function of amount of added boronic acid.

**Reaction with pyrogallol**: Owing to the quick oxidation of this electron-rich polyol, TCEP was added to the stock solution of pyrogallol in a molar ratio pyrogallol/TCEP 1:1.5. Due to the decrease in pH following TCEP addition, a few drops of 0.1 N NaOH were added to the pyrogallol stock solution until pH = 7.4.

**Reaction with ascorbic acid**: Due to the acidic nature of this diol, 1M PBS buffer was used to raise the pH to 7.4.

**Reaction with quercetin**: Owing to the very poor solubility of quercetin in a water environment a Tween 20 (a oligo(ethylene glycol) (polyoxyethylene) derivative of sorbitol monolaurate widely employed in bioassays and pharmaceutical formulations) was used. For quercetin concentrations up to 1 mM a 1:1 Tween/quercetin molar ratio was sufficient to completely solubilise it in 0.1 M PBS buffer, since its UV-Vis absorbance reaches a plateau and does not increase with larger amounts of surfactant. The highest concentration of Tween 20 used is limited by its ability to hamper the binding reaction. A maximum of 8% w/w of Tween 20 can be used without affecting the reaction kinetics.

**Reaction with polymeric derivatives**: Typical experiments were performed with HA-Dopamine (P2), HA-Veratrylamine (T2) and PEG-Dopamine using the above mentioned technique with both 3-APBA and HA-3-APBA. The molar ratio of boronic/diol was restricted to 1:2.5 to prevent highly viscous polymeric solutions.
**Enzymatic Reactions.** Typical experiments were performed in 96 well plates at 37 °C sealed with the help of PCR plate adhesive seals to prevent evaporation; three repetitions were carried out per sample in 0.1M PBS buffer.

- **Hydroxylation of estradiol.** Estradiol can bind to boronic acid only upon the introduction of a second alcoholic function, which can be carried out by cytochrome P450 variant 1A2. Estradiol is barely soluble in the water medium that we have used for the diol/boronic reaction (0.1 M PBS) and thus requires a solubilising agent; since most surfactants would impair the activity of cytochrome P450, we have employed β-cyclodextrin sulphobutylether, (i.e an anionic, very water soluble cyclodextrin: neutral cyclodextrins did not provide sufficient solubilisation, while most surfactants do) in 1:1 molar ratio with estradiol. A stock solution of CYP1A2 was prepared by diluting 30 µL of a 1000 pmol/mL solution with 375 µL of 0.1 M PBS. To this CYP solution, 7.8mg of NADPH was added for its activation. 48 µL of ARS solution (final concentration: 0.56 mM) was added to each well, followed by 55 µL of HA-3-APBA (final concentration: 0.82 mg/mL, 0.56 mM boronic moiety); the mixture was allowed to react for 30 seconds (stable change in colour from red to orange). On completion of this reaction, a calculated amount of estradiol (HA-3-APBA : estradiol molar ratios ranging from 1:0.1 to 1:4.5) was added to each well, followed by 27 µL of the CYP1A2 stock solution (corresponding to 2 pmoles of CYP1A2 calculated from the stock solution of 74 pmol/mL + 2.5mM final concentration of NADPH).

- **Demethylation of 3-Methoxytyramine.** 3-methoxytyramine can bind to boronic acid only upon removal of its methoxy group, which can be carried out by cytochrome P450 variant 2D6. A stock solution of CYP2D6 was prepared by diluting 20 µL of a 500 pmol/mL solution with 520 µL of 0.1 M PBS. To this CYP solution, 8.34mg of NADPH was added which is needed for activation of the enzyme. The reaction of ARS with HA-3-APBA was conducted as described above. Appropriate amounts of 3-methoxytyramine, ranging from a HA-3-APBA : 3-methoxytyramine molar ratio of 1:0.5 to 1:3.2, were then added to each well, followed by 27 µl of the CYP2D6 stock solution (corresponding to 0.5 pmoles of CYP2D6 calculated from the stock solution of 18.5 pmol/mL + 2mM final concentration of NADPH).
3.3.7 Reactions between polymeric species

A typical experiment involved the use of a 30ml glass vial with HA derivatives employed at a concentration of 3mg/ml in 0.1M PBS with a total volume of 26.7ml. The reaction was assisted by continuous stirring for 1 hour.

For the linkage of HA-3-APBA to dopamine, 13ml of a stock solution of HA-3-APBA (X1, 17% D, 0.00125M boronic) was first added followed by 5.85ml of dopamine solution (0.0125M, 3-APBA: dopamine molar ratio = 1:10). TCEP (0.0188M, Dopamine: TCEP molar ratio = 1:1.5) was added to the solution to prevent oxidation of dopamine.

For the linkage of HA-Dopamine to 3-APBA, 6.67ml of a stock solution of HA-Dopamine (P2, 16% D, 0.00116M dopamine) was first added followed by 2.83ml of 3-APBA solution (0.0116M, Dopamine: 3-APBA molar ratio = 1:10) and finally TCEP (0.00174M, Dopamine: TCEP molar ratio = 1:1.5) was added to the solution.

Purification for both the samples involved dialysis using 3500 Da molecular weight cutoff membranes against water containing dissolved sodium bicarbonate at pH 7.4. On completion of dialysis (monitored by conductivity) the samples were freeze dried and then evaluated for $M_w$, $R_g$ and $A_2$ using SLS.
3.4 Results and Discussion

3.4.1 Preparation of HA derivatives

The functionalisation of HA with amine-containing molecules can be easily performed using water-soluble derivatives of carbodiimide (EDC) and N-hydroxysuccinimide (sulpho-NHS). This popular literature procedure\textsuperscript{33, 34} is based on the conversion of HA carboxylate groups into rather unstable O-acylisoureas, which can also react with alcohols in the HA structure, giving rise to branching and cross-linking\textsuperscript{35}. The O-acylisoureas are thus transformed into amine-selective NHS esters, which in this study have allowed the functionalisation of HA in good yields, as witnessed by \textsuperscript{1}H-NMR (Figure 3-1) and UV spectra (Figure 3-2).

There are a number of pathways in which this reaction proceeds leading to both favourable and side products (Scheme 3-3) depending on the amount and type of reactants used. The first step is the reaction of the carbodiimide, EDC, with a proton to form a carbocation, which in the absence of a dissociated carboxylic acid, hydrolyses to form a urea derivative. This is the main reason for using a freshly prepared solution of EDC\textsuperscript{36}. In the presence of dissociated carboxylic acid, the carbocation can react with an ionized carboxyl group to form O-acylisourea. Depending on the presence or absence of nucleophile at this stage, different scenarios are possible.

In the absence of a nucleophile, the O-acylisourea would reprotonate at the site of the Schiff base, and change into a carbocation which can partially hydrolyse to form urea or be attacked by the various bases which are present. Since an ionized carboxylic acid group is a very strong base, it can react with the carbocation to form carboxylic anhydride if the carboxyl group is cyclizable, which can readily react with amines, to form the corresponding amide. However, since the carboxyl group of HA is noncyclizable, the carbocation would react with the unionized amine to form the amide bond and with a water molecule to form carboxylate. Since the concentration of water is much higher in the system and most of the molecules of the amine are in the ionized form, this would lead to most of the carboxylation forming the carboxylate, and hence giving a very low yield. This is the main reason for not using EDC on its own without a nucleophile. Also, the presence of excess carbodiimide, would favour the reaction of the carbocation with the excess carbodiimide to form N-acylurea as a byproduct which is unreactive towards primary amines and would be covalently attached to HA\textsuperscript{37}.
In the presence of a nucleophile, such as NHS (we have used sulfo-NHS since it has good water solubility), the dissociated hydroxyl group of the NHS makes a nucleophilic attack on the O-acylisourea to form a succinimidyl ester which is more stable towards hydrolysis, and a urea derivative. The formation of N-acylurea is not possible since the succinimidyl ester cannot undergo N→O displacement. This succinimidyl ester is then attacked by a non-dissociated primary amine (dopamine, 3-APBA or veratrylamine) forming the amide bond and regenerating NHS.

Scheme 3-3. Carboxidiimide mediated synthesis process of HA derivatives and the side reactions involved. The reaction of EDC with carboxylate groups leads to various side reactions depending on the presence or absence of a nucleophile such as formation of urea derivatives and re-formation of the carboxylate group.
The ratio between reactands influenced the functionalisation yield of HA with dopamine, although the use of large excess of promoters or amine did not necessarily correspond to an increase in the molar fraction of functionalised units (Table 3-1). Three points are evident from Table 3-1: the first that any increase in the molar ratio of EDC : Sulpho-NHS (1:1.5 – 1:2) does not vary the yield which means that the excess nucleophile present does not have any positive effect; the second point is that any increase in the molar ratio of EDC : amine (>1:2) does not necessarily show an increase in the yield as well; and the final point is that an increase in the COO⁻ : EDC molar ratio (>1:2) shows no change in the yield (13-15%).
Figure 3-1. $^1$H-NMR spectra of (a) HA (1.1 MDa, bottom), EDC and Sulpho-NHS. (b) HA (1.1 MDa), dopamine and four HA-dopamine derivatives – P1, P2 and two others which have different molar ratios with excess reactants. (c) HA (1.1 MDa), 3-APBA, X1, X2, veratrylamine, T1 and T2. The spectra of the functional derivatives are always better resolved than that of the parent polymer. For example, the peak at 4.3 – 4.5 ppm (H-1 of GlcA) is very broad and almost invisible for HA, while it is clearly visible for all derivatives. We ascribe the better resolution of the latter spectra to the marked reduction in molecular weight during the functionalisation reaction. Reference to the numbering of carbon atoms is given in the experimental part, interpretation of NMR spectra.

Therefore, two reaction conditions were selected, which offered a good compromise between high yield and low amounts of reactants; they were then adopted also for the reactions of HA with the other two amines (veratrylamine and 3-APBA), which exhibited considerably higher conversions than dopamine: 16 or 17% vs. 8.5% (polymers T1, X1 and P1, respectively, see Table 3-1 and Figure 3-1) for one set of conditions and 25 and 27% vs. 16% for the other (polymers T2, X2 and P2, respectively). The better reactivity may be due to the presence of ascorbic acid in the reaction with dopamine: the acidity of the reducing agent may partially protonate the primary amine, reducing its reactivity. It is worth pointing out that the reactions of HA
with dopamine carried out in the absence of ascorbic acid turned black in a few hours, as a consequence of the oxidation and polymerization of catechol groups (Scheme 3-4).

\[
\text{OH} \quad 1/2 \text{O}_2 \quad \text{OH} \quad \text{O}_2 \quad \text{OH} \quad \text{OH} \\
\text{catechol} \quad \text{o-benzoquinone} \\
\]

**Scheme 3-4.** Oxidation of catechol to o-benzoquinone.

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**Figure 3-2.** UV spectra of the HA derivatives (1 mg/mL): HA-dopamine (P1 - 8.4%, P2 - 16%), HA-veratrylamine (T1 - 16%, T2 - 25%) and HA-3-APBA (X1 - 17%, X2 - 24%) showing the maximum absorbance at 280 nm for dopamine, 277 nm for veratrylamine and 296 nm for 3-APBA. Reference to the calculation for the degree of derivatisation is given in the experimental part, interpretation of UV spectroscopy.

Figure 3-1 (a) shows the $^1$H-NMR spectra for HA (1.1 MDa) along with the spectra for the reactants EDC and sulpho-NHS. This provides a basis for comparing the spectra of the HA-derivatives presented in Figure 3-1 (b) and (c) to ensure purity and complete removal of excess reactants. As mentioned earlier, for the reaction of HA with
dopamine, various molar ratio of reactants were tested, and the spectra of few of them have been showed in Figure 3 (b) which clearly show from the integrals, the degree of derivatisation obtained. We selected the acetyl group of HA for comparing the integral as it has its own individual distinct peak and it does not participate in the reaction which ensured that its value is unchanged before and after the reaction. For the integral of dopamine, we selected the 3 hydrogen protons present on the aromatic ring since, they are distinct and do not interfere with any peak of HA.

There is a substantial increase in the derivatisation percentage between P1 (molar ratio COO\(^{-}\):EDC = 1:0.5) and P2 (molar ratio COO\(^{-}\):EDC = 1:2), and this is owing to the quadruple increase in the amount of EDC, sulpho-NHS and dopamine. This is also seen in the UV spectra (Figure 3-2 shows the UV spectra for samples P1 and P2 at 1 mg/mL) and the degree of derivatisation obtained from the UV spectra (using the equation provided in section 3.3.2, UV spectroscopy) corresponds to the values obtained by NMR. However, on increasing the reactants any further, the integral do not seem to increase in the same manner, which might be due to the side reaction which leads to the reforming of the carboxylate groups.

Figure 3-1 (c) shows the \(^1\)H-NMR spectra for HA-3-APBA and HA-veratrylamine derivatives along with their respective reactants. In the case of 3-APBA, we have selected the 4 hydrogen protons present on the aromatic ring since they are in the region of 7 – 8 ppm and do not interfere with the peaks of HA. We can see a clear distinction between the integrals of X1 and X2 owing to the increase of reactants. In the case of veratrylamine as well, we have selected the 3 hydrogen protons present on the aromatic ring, and have not opted for the aliphatic spacer or the methyl groups since they seem to be shadowed by the peaks of HA; and a very clear difference is visible in the derivatisation percentages of T1 and T2. The degree of derivatisation obtained in both these cases is also comparable to the values obtained using UV spectroscopy (Figure 3-2, samples X1, X2, T1 and T2).

A common thing which is visible in all the spectra is the absence of the side product urea (usually present around 1 ppm), as well as any excess EDC or sulpho-NHS which have been removed during dialysis.

It must be noted that all reactions determined a clear decrease in HA molecular weight, as shown by static light scattering (Table 3-2). A huge difference in the \(M_w\) of the HA derivatives is noticeable as compared to the parent HA which is common due to degradation of the HA during the reaction with EDC and sulpho-NHS. One main point
is that between the three HA derivatives (P2, T1 and X1) having the same degree of
derivatisation, the $M_w$ is similar which denotes that the degradation of HA is
independent of the type of primary amine used for the reaction. Literature suggests that
HA can be hydrolysed by acids and bases and is more sensitive towards bases. The
degradation rate constant at pH 13 is approximately 50 times larger than at pH 2.
Therefore, it is highly probable that base-catalysed hydrolysis cleaves the 1→4 and/or
1→3 glycosidic bond of HA, resulting in low-molecular fragments as seen in Table 3-
238-40.

The decrease in $M_w$ has caused a decrease in $R_g$ as well but it is not directly
proportional, as a tenfold decrease in the $M_w$ of HA has caused only a twofold decrease
in the $R_g$. An $R_g$ of 111.2 ± 10 nm for HA of 1 million g/mol is in accordance with
literature values of 120 nm for 1 – 1.2 million g/mol. Also an $R_g$ of 55 ± 5 nm for all the
HA derivatives (P2, T1 and X1) is in accordance with literature values of 55 nm for
0.236 million g/mol41.

The second virial coefficient is a property describing the interaction strength between
the molecule and the solvent. For samples where $A_2 > 0$, the molecules tend to stay in
solution. When $A_2 = 0$, the molecule-solvent interaction strength is equivalent to the
molecule-molecule interaction strength and the solvent is described as being a theta
solvent. When $A_2<0$, the molecules will tend to crystallize or aggregate. In our case, all
the $A_2$ values (for HA and its derivatives) are positive indicative of intermolecular
repulsion42.

A decrease in the molecular weight of HA usually reduces the $A_2$ denoting a
destiffening and contraction of the coil which is seen clearly in the derivatisation of
HA43. It has been suggested that this might be related to the accessibility and number of
hydrogen bond forming groups as HA molecules in solution are in a dynamic process of
forming and breaking of hydrogen bonds involving the $C_2$ hydroxyl group of glucuronic
acid, the acetamido oxygen of N-acetylglucosamine and the oxygen of carboxylic
groups of a second glucuronic acid. These interactions in high molecular weight HA
might be intramolecular due to the large volume occupied by the molecule and physical
entanglements of the chain; whereas with a decrease in the molecular weight, the
volume occupied by each chain decreases and the molecules begin to experience
intermolecular interactions which compete with intramolecular ones, resulting in a
decrease in $A_2$42.
The values of $A_2$ for P2 and X1 presented in Table 3-2 are in accordance with the literature values for similar molecular weight pure HA which denotes that the derivatisation does not hamper the conformational properties of the HA\textsuperscript{43, 44}. The $A_2$ for T1 seems to be more of an anomaly since we do not expect it to be much different from the other HA derivatives.

The detailed discussion of the complexes (P2/3-APBA and X1/dopamine) has been presented in section 3.4.3 Part B (Evaluation of boronic-diol equilibria on HA derivatives).

Table 3-2. Results of static light scattering measurements: weight average molecular weight ($\bar{M}_w$), radius of gyration ($R_g$) and second virial coefficient ($A_2$) for HA, its functional derivatives\textsuperscript{a} and their complexes.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Functionalisation (mol %)</th>
<th>$\bar{M}_w$ (g/mol)</th>
<th>$R_g$ (nm)</th>
<th>$A_2$ (cm$^3$/mol/g$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>0</td>
<td>1.02 ± 0.22×10$^6$</td>
<td>111.2 ± 10</td>
<td>4.60 ± 0.07×10$^{-3}$</td>
</tr>
<tr>
<td>P2</td>
<td>16</td>
<td>1.86 ± 0.27×10$^5$</td>
<td>55.7 ± 5.2</td>
<td>2.30 ± 0.21×10$^{-3}$</td>
</tr>
<tr>
<td>T1</td>
<td>16</td>
<td>2.34 ± 0.28×10$^5$</td>
<td>54.0 ± 5.3</td>
<td>8.17 ± 0.42×10$^{-3}$</td>
</tr>
<tr>
<td>X1</td>
<td>17</td>
<td>2.74 ± 0.30×10$^5$</td>
<td>55.8 ± 2.4</td>
<td>2.62 ± 0.15×10$^{-3}$</td>
</tr>
<tr>
<td>P2/3-APBA</td>
<td>=</td>
<td>1.08 ± 0.09×10$^5$</td>
<td>30.0 ± 6.6</td>
<td>2.11 ± 0.09×10$^{-3}$</td>
</tr>
<tr>
<td>X1/dopamine</td>
<td>=</td>
<td>2.22 ± 0.14×10$^5$</td>
<td>38.7 ± 8.0</td>
<td>3.15 ± 0.07×10$^{-3}$</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All the polymers showed statistically indistinguishable results.

3.4.2 Evaluation of degradability and cytotoxicity

An ideal bioconjugate should show negligible cytotoxicity and should also be either excreted or degraded at the end of its therapeutic action, in order to avoid possible problems from long-term accumulation. HA is inherently non-cytotoxic, but it is rapidly enzymatically degraded \textit{in vivo}: its half-life time can be as low as half a day\textsuperscript{10}. An ideal HA-based bioconjugate should maintain the biocompatibility and degradability, however reducing the rate of the latter. This is, however, a rather general effect: the introduction of functional units on the carboxy groups is indeed expected to decrease the rate of enzymatic degradation, as it happens for a number of HA esters, such as the
HYAFF family. Mammalian hyaluronidases bind to HA utilizing its anionic carboxylic groups, with a binding region spanning at least an hexasaccharide sequence; the introduction of even few non-anionic groups sharply decreases the number of all-ionic hexasaccharidic sequences, thus dramatically affecting the degradability of HA derivatives.

Here we have compared the degradability and cytotoxicity of derivatives containing boronic acid (X1 and X2) to those of dopamine derivatives (P1 and P2), using veratrylamine ones (T1 and T2) to differentiate effects deriving from the aromatic nature of catechols from those of REDOX origin.

We have examined the effect of hyaluronidase (from ovine testes: broad spectrum hydrolase catalyzing the cleavage of the (1→4) glycosidic link) through a semi-quantitative but rapid test, monitoring the viscosity of the water solutions of HA derivatives. All of them showed a significant response, with a marked decrease of the viscosity (see appendix, Figure 3-6).

We here have employed two different parameters for a comparison of the degradability of the polymers (Figure 3-3).

A) Using the rather rough assumption that the decrease in viscosity during degradation is mostly related to a reduction in molecular weight, we have employed the ratio between initial and plateau viscosity $\eta_{rel}(0)/\eta_{rel}(\infty)$ as an indication of the maximum extent of enzymatic degradation. With the exception of T1 (16% veratrylamine, statistically indistinguishable from HA), the derivatives showed a markedly lower extent of degradation than their parent polymer, decreasingly with increasing functionalisation.
**B)** The degradation kinetics of the polymers more closely reflects the efficiency of enzyme/substrate binding. Fitting the viscosity data with a simple exponential kinetic model, we have calculated a “normalized reactivity” (see experimental section), a sensitive parameter exhibiting a reduction of up to almost two orders of magnitude upon functionalisation. For veratrylamine and 3-APBA, similar percentages of functionalisation similarly affected the “normalized reactivity”, decreasing with increasing amounts of functional groups. The presence of dopamine, on the contrary, appeared to cause a dramatic reduction in degradation rate also at 8.5 mol % of functional groups, which we can possibly ascribe to the REDOX reactivity of catechols.

The cytotoxicity of the HA derivatives was then evaluated utilizing the MTS test of metabolic activity on L929 fibroblasts and J774 macrophages. Macrophages are characterized by higher endocytic activity than fibroblasts, and produce larger amounts of the main receptor for HA internalization (CD44)\(^47\); it was therefore expected that they offer a more sensitive model for the cytotoxicity of HA-based systems. However, no sound difference was recorded between the two cell lines (Figure 3-4). Furthermore, some moderate negative effects on cell viability were recorded only for dopamine-containing polymers (Figure 3-4); with both cell lines, these polymers showed IC50
values in the range of 1-3 mg/mL, with no apparent reduction in cell viability below 0.5 mg/mL. The other HA derivatives showed even less cytotoxicity (IC50 > 5 mg/mL or simply not recorded in the range of concentrations evaluated, i.e. above 1% wt.), which was particularly low for the 3-APBA derivatives. It appears therefore that the methylation of OH groups has significantly increased the tolerance of both cell lines to the presence of catechols.

Figure 3-4. Viability of fibroblasts (left) and macrophages (right) after 24 hours of exposure to different concentrations of the HA derivatives.

The boronic acid derivatives showed a very favourable toxicity profile; they also preserved significant degradability, while their slower kinetics in comparison to the parent polymer would be beneficial in the perspective of a prolonged circulation. We have therefore focused on HA-boronic derivatives, and specifically on X2 for further investigations.

Comparing the degradability and the cytotoxicity results, we come to the conclusion that in general, the higher the derivatisation percentage, the less favourable is the derivative as its degradability decreases and its cytotoxicity increases. The tests for HA-dopamine derivatives showed that they had the worst degradability and were the most cytotoxic as compared to the other derivatives; this is mostly due to their oxidation potential. The HA-veratrylamine derivatives showed a better degradability (especially T1) and cytotoxicity profile than the HA-dopamine derivatives, owing to the methylation of the diol, while the HA-3-APBA derivatives showed an equivalent degradability but the best cytotoxicity profile. One of the main aims of synthesising a
HA based polymeric carrier which has good degradability, longer circulation time and least cytotoxicity has been achieved with the HA-3-APBA derivatives.

### 3.4.3 Evaluation of boronic-diol equilibria on HA derivatives

The boronic/diol equilibrium constants were obtained by utilizing a previously developed method based on the batochromic shifts in the absorbance spectrum of Alizarin red S (ARS), which is a diol-containing dye with an absorption peak at 519 in a free form that shifts to 480 in the boron-complexed form. The binding equilibrium of ARS to a boronic acid-containing compound is governed by a constant $K_1$, which is readily calculated from the hypsochromic shifts of ARS. Using 3-APBA and the HA derivative X2, we have obtained $K_1 = 5150 \pm 200 \, M^{-1}$ for ARS – APBA and of ARS-HA-BA ($K_1$) is $2550 \pm 150 \, M^{-1}$. When exposing these two complexes to other diols, a competitive equilibrium is established and from the resulting batochromic shift of ARS it is possible to calculate the constant $K_2$ for the binding of the boronic acid to the second diol. From the results provided in Table 3-3 it is possible to highlight the following points:

**A)** We have used three negative controls: phloroglucinol, which has three aromatic non-vicinal OH groups; veratrylamine and T2, which feature methylated catechol units. As it happens with 3-APBA, no binding was recorded also for X2, ensuring therefore that the polymeric HA structure does not directly interfere in the equilibrium.

**B)** The boronic/diol complexation has an effect on the macromolecular dimensions of HA derivatives. A distinct shrinkage ($R_g$ reduced from around 55 nm to less than 40 nm) was recorded both for P2 reacting with 3-APBA and for X1 reacting with dopamine (Table 3-2, bottom). Upon formation of the boronic/diol complex, the number of polymer-bound anions increase, due to the quaternarised boron atoms; the modest protonation of dopamine or 3-APBA at neutral pH does not counterbalance this effect. Therefore, on the grounds of the increased electrostatic repulsion between chain segments, one would expect an expansion of the HA coil, rather than its shrinkage.
C) With the exception of ARS, all diols bind more strongly to the macromolecular derivative $X_2$ than to the low molecular weight compound 3-APBA. On the other hand, generally one would expect the polymer matrix to decrease the binding strength at least due to steric hindrance.

D) In two cases, catechols were generated enzymatically from non-reacting precursors: respectively, 2-hydroxyestradiol from estradiol and dopamine from 3-methoxytyramine. The kinetics of the two enzymatic reactions, respectively an hydroxylation and a demethylation, can be followed through the competitive equilibrium of the newly formed diols with the ARS/3-APBA and ARS*X2* complexes (see appendix, Figure 3-7). The calculation of the kinetic parameters ($v_{\text{max}}$ and $K_M$) is based on the assumption that the complexation is considerably quicker than the enzymatic reactions; therefore, a slow boronic/diol complexation would provide different values of the kinetic parameters of the enzymatic reactions. In our case (Table 3-4) the use of 3-APBA or $X_2$ as reporters for the enzymatic reactions were indistinguishable, suggesting the kinetics of the complexation not to be heavily affected by the presence of the macromolecular chain.

E) Despite both reagents being polymeric and multifunctional, in the complexation between P2 and $X_2$ no gelation or increase in viscosity could be recorded (see appendix, Figure 3-8).

In summary, the macromolecular HA backbone does not participate to the boronic/diol complexation, but does not hinder it and even appears to favour it. The reduction in the dimension of the macromolecular coil could indicate the presence of attractive interactions between boronic esters and macromolecular backbone that counterbalance and possibly overwhelm the increased electrostatic repulsion between the anions. The absence of rheological effects of the complexation between polymeric partners can also be interpreted along these lines, with a collapse of the macromolecular structures around the boronic esters that will favour strong aggregation of a very limited numbers of partners rather than weaker aggregation involving several macromolecules. A possible mechanistic explanation of this effect is that the HA backbone may cause a decrease in the $pK_a$ of the boronic acids. The opposite effect has been observed in synthetic polymers such as poly(N-isopropyl acrylamide)$^{48}$, where the decrease in
acidity is related to the closer proximity among boronic acids. However, to our knowledge no literature report exists about the acidity of boronic acids linked to strongly H-bonding polymeric substrates, such as HA. We have used an empirical equation validated by Wang\textsuperscript{49}, which sets the optimal pH as the numerical average between the pK\textsubscript{a} of diol and boronic acid, and calculated optimal pH values for 3-APBA and for X2, with the latter pK\textsubscript{a} calculated to be 0.5 units lower than that of 3-APBA (Table 3-3, 5\textsuperscript{th} and 7\textsuperscript{th} column). Following this, the optimal pH for all diols except ARS, would be closer to neutrality for X2, i.e. they would bind the HA derivative more strongly than 3-APBA. The reverse applies for the more acidic diols. Indeed this is what is experimentally recorded for ARS and all other diols.

Using the estimated pK\textsubscript{a} value of 8.4 from the titration plot of X2 (see appendix, Figure 3-9), we believe that due to the increased acidity and therefore increased reactivity of HA-bound boronic acids, it is possible to rationalize all our experimental observations. Comparing the results from Table 3-3 and Table 3-4, we come to the following conclusions: HA-boronic derivative X2 binds to 1,2-diols better than 3-APBA due to a lower pK\textsubscript{a}, which brings the optimal pH of binding to most diols closer to physiological pH. This is an excellent finding, as X2 is reasonably reactive towards diols, and does not affect the binding strengths negatively due to the presence of the polymeric chain. It also signifies that, since the optimal pH of binding is closer to physiological pH, the release of the diols would be at a less acidic pH than that of the diols bound to 3-APBA. The enzymatic reactions also show that the polymeric chain does not hamper the reactivity of the enzyme towards the substrates, and proves that the analytical method works in the case of polymeric chains as efficiently as it does for small molecules. The binding of X2 with other polymeric-diols proves that our carrier could be used for transporting big molecules as well; however studies with proteins and other big molecules have not been performed. This helps us achieve one the main aims of the project, which was to study the conjugation of diols to our polymeric carrier in order to ensure a decent reactivness towards diols.
Table 3.3. Equilibrium constants ($K_1$ for ARS and $K_2$ for all other diols) for various diols with HA-BA and 3-APBA\textsuperscript{a} at pH 7.4 in 0.1 M PBS buffer. SD are calculated over $n = 3$. The values of optimal pH for binding are calculated using the 3-APBA actual pK\textsubscript{a} and the estimated pK\textsubscript{a} of X2 to be 8.4.

<table>
<thead>
<tr>
<th>Class</th>
<th>Name</th>
<th>pK\textsubscript{a}</th>
<th>$K$ (M\textsuperscript{-1})</th>
<th>Opt. pH\textsuperscript{b}</th>
<th>$K$ (M\textsuperscript{-1})</th>
<th>Opt. pH\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARS</td>
<td>4</td>
<td>5150 ± 200</td>
<td>6.5</td>
<td>2550 ± 150</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>Negative controls</td>
<td>Phloroglucinol 8.5\textsuperscript{50}</td>
<td>0</td>
<td>8.7</td>
<td>0</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Veratrylamine</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>Aromatic diols</td>
<td>Pyrocatechol 8.62\textsuperscript{51}</td>
<td>1490 ± 70</td>
<td>8.8</td>
<td>1844 ± 110</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dopamine 8.89\textsuperscript{52}</td>
<td>1555 ± 66</td>
<td>8.9</td>
<td>1955 ± 117</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dopamine from 3-methoxytyramine 8.89\textsuperscript{52}</td>
<td>1550 ± 73</td>
<td>8.9</td>
<td>1839 ± 110</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epinephrine 8.55\textsuperscript{53}</td>
<td>1445 ± 65</td>
<td>8.7</td>
<td>1910 ± 114</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Norepinephrine 8.6\textsuperscript{54}</td>
<td>1545 ± 66</td>
<td>8.8</td>
<td>1969 ± 118</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-hydroxy estradiol from estradiol 9.4\textsuperscript{55}</td>
<td>955 ± 38</td>
<td>9.2</td>
<td>1061 ± 67</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>Aromatic polyols</td>
<td>Pyrogallol 9.1\textsuperscript{56}</td>
<td>1796 ± 78</td>
<td>9.0</td>
<td>2325 ± 140</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quercetin 7.6\textsuperscript{57}</td>
<td>1240 ± 98</td>
<td>8.3</td>
<td>1312 ± 115</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Sugar diols</td>
<td>Mannose 12.13\textsuperscript{58}</td>
<td>5.85 ± 1.2</td>
<td>10.5</td>
<td>6.43 ± 0.85</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose 12.3\textsuperscript{58}</td>
<td>3.75 ± 0.4</td>
<td>10.6</td>
<td>3.98 ± 0.31</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid 11.8\textsuperscript{59}</td>
<td>6.4 ± 0.94</td>
<td>10.4</td>
<td>7.04 ± 0.83</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>Polymetric diols</td>
<td>P2 (8.9)\textsuperscript{5}</td>
<td>1440 ± 61</td>
<td>(8.9)</td>
<td>1717 ± 103</td>
<td>(8.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PEG-Dopamine (8.9)\textsuperscript{5}</td>
<td>1025 ± 42</td>
<td>(8.9)</td>
<td>1432 ± 86</td>
<td>(8.6)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} with the exception of P2, T2 and PEG-dopamine, all the 3-APBA were obtained in a previous study.
the optimal pH was calculated according to Yan et al. as \[ pH_{opt} = \frac{pK_a(boronic) + pK_a(diol)}{2} \]

c in the presence of sulphonated β-cyclodextrin to solubilise the steroid. The end point of the enzymatic reaction was used to calculate the value of the equilibrium constant, assuming complete conversion of estradiol.

d in the presence of TCEP to avoid pyrogallol oxidation.

e in the presence of Tween 20 to solubilise quercetin.

f in 1 M PBS.

g due to the structural similarity, we estimate the pK\(_a\) of this catechol groups to be substantially analogous to that of dopamine.

**Table 3-4.** Comparison of the kinetic parameters for the CYP2D6-mediated demethylation of 3-methoxymetramine and for the CYP1A2-mediated 2-hydroxylation of estradiol, using the complexation of the products to 3-APBA and X2 as a reporter of the enzymatic reactions.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reporter</th>
<th>( K_M ) (µM)</th>
<th>( v_{max} ) (pmol/pmol P450/min)</th>
<th>Hydrolase activity (CLint) (^a) (µL/pmol P450/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>3-APBA</td>
<td>3800 ± 650</td>
<td>65 ± 13</td>
<td>0.017</td>
</tr>
<tr>
<td>Estradiol</td>
<td>X2</td>
<td>3570 ± 990</td>
<td>56 ± 11</td>
<td>0.015</td>
</tr>
<tr>
<td>3-methoxymetramine</td>
<td>3-APBA</td>
<td>281 ± 8</td>
<td>49 ± 4</td>
<td>0.18</td>
</tr>
<tr>
<td>3-methoxymetramine</td>
<td>X2</td>
<td>285 ± 6</td>
<td>54 ± 3</td>
<td>0.19</td>
</tr>
</tbody>
</table>

\(^a\) CLint (intrinsic clearance = \( v_{max} / K_M \)) is a common measure of the enzymatic activity in the cell.
3.5 Conclusions

In an attempt to evaluate the feasibility of the use of boronic/diol complexation for the
purpose of bioconjugation, we have synthesised and conducted a semi-quantitative
evaluation of the toxicity and degradability of boronic acid- or catechol-containing
derivatives of hyaluronic acid. The more favourable toxicity led us to prefer the use of
the first ones. We have then further compared the performance of a boronic acid-
containing HA derivative (X2, 25% mol of carboxy groups bearing phenylboronic acid
residues) to that of the low molecular weight 3-APBA in the complexation to a library
of diols. Rather surprisingly, the presence of this macromolecular backbone did not
hamper the complexation, which could have happened either because of steric hindrance
or of increased proximity between mutually repelling anionic boronic esters. On the
contrary, the equilibrium constants to the vast majority of diols slightly increased (5-
15%) and clear reductions in the macromolecular size were recorded too. We have
attributed these effects to an increase in the acidity of the boronic acids, which
rationalize the results in terms of differences of the optimal pH for binding.
The negligible toxicity, reasonable degradability and good binding strength (Kₐ around
500 µM for catechols) are encouraging in the perspective of applying HA-boronic acids
as carrier structures for the delivery of diol-functionalised payloads.
3.6 Appendix

Table 3-5. Raw Data for Refractive Index of HA and its derivatives used for the calculation of dn/dc.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Refractive index for concentration of</th>
<th>dn/dc&lt;sup&gt;a&lt;/sup&gt; (mL/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 mg/mL</td>
<td>2.2 mg/mL</td>
</tr>
<tr>
<td>HA</td>
<td>1.33488</td>
<td>1.33472</td>
</tr>
<tr>
<td>X1, X1/dopamine</td>
<td>1.33485</td>
<td>1.3347</td>
</tr>
<tr>
<td>T1</td>
<td>1.33411</td>
<td>1.33399</td>
</tr>
<tr>
<td>P2</td>
<td>1.33465</td>
<td>1.33457</td>
</tr>
<tr>
<td>P2/3-APBA</td>
<td>1.33452</td>
<td>1.33445</td>
</tr>
</tbody>
</table>

<sup>a</sup> A plot of RI (y-axis) vs Concentration in g/mL (x-axis) gives a straight line, the slope of which is the dn/dc.

Figure 3-5. Zimm Plot for parent HA (left) and for P2 (right).
Chapter 3

Figure 3-6. *Left*: typical behaviour of the dynamic viscosity of HA and HA derivatives as a function of time upon exposure of water solutions. The concentration of the solutions were adjusted to obtain comparable values of the dynamic viscosity prior to degradation: HA, 1 mg/mL; P1, 16 mg/mL; P2, 18 mg/mL; T1, 3 mg/mL; T2, 16 mg/mL; X1, 5 mg/mL; X2, 9 mg/mL. *Right*: values obtained from exponential fits of the viscosity curves (averages over three samples).

<table>
<thead>
<tr>
<th>opam</th>
<th>Sample</th>
<th>$\eta_0/\eta_3$</th>
<th>1/r (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td></td>
<td>22.62</td>
<td>1.2</td>
</tr>
<tr>
<td>P1 (dopam, 8.5%)</td>
<td></td>
<td>9.58</td>
<td>0.051</td>
</tr>
<tr>
<td>P2 (dopam, 16%)</td>
<td></td>
<td>6.08</td>
<td>0.074</td>
</tr>
<tr>
<td>T1 (verat., 16%)</td>
<td></td>
<td>28.27</td>
<td>0.21</td>
</tr>
<tr>
<td>T2 (verat., 25%)</td>
<td></td>
<td>13.38</td>
<td>0.041</td>
</tr>
<tr>
<td>X1 (3-APBA, 17%)</td>
<td></td>
<td>8.73</td>
<td>0.078</td>
</tr>
<tr>
<td>X2 (3-APBA, 24%)</td>
<td></td>
<td>4.52</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Figure 3-7. Michaelis-Menten plots for the two enzymatic reactions followed in this study, using the complexation of the catechol product with 3-APBA or X2 as a reporter. The dashed line shows the result of a hyperbolic fitting on the X2 data. *Left*: demethylation of 3-methoxytyramine. *Right*: hydroxylation of estradiol.
Figure 3-8. The storage ($G'$) and loss ($G''$) moduli of a mixture of X2 and P2 (both 5 mg/mL) in 10 mM PBS (frequency 1 Hz) as a function of time after mixing. No sign of increase in viscosity or build-up of an elastic character can be seen and the solution shows no sign of significant aggregation.

Figure 3-9. pK<sub>a</sub> determination for X2 using 10 mM NaOH
3.7 References

4. Conclusion

**Aims and Hypotheses.** The two main aims of this thesis were to study the complexation of independent and polymer-conjugated boronic acids with diols in simple and complex systems (including enzymatic systems) using the batochromic shifts of the optical reporter ARS, and to prepare suitable HA derivatives for using boronic/diol complexation as a bioconjugation reaction. The hypothesis of this project is that any non-toxic, non-immunogenic, non-excretable and biodegradable polymer like HA is capable of being transformed into a drug delivery carrier by conjugation with a boronic acid, and can be used to deliver diol containing or catechol-containing/conjugated drugs/peptides/proteins by boronic-diol reversible complexation chemistry. Once injected into the body, the high molecular weight polymer would allow for a long circulation time and/or solubility of the drug, while possibly keeping it inactive while bound. The conjugate would then undergo endocytosis using cell surface HA receptors and, due to the reversible nature of the boronic-diol complexation chemistry, the acidic pH of the late endosome or lysosome of tumours and inflammatory tissues would release the drug. The HA-boronic would then undergo degradation by hyaluronidase enzymes. This would allow for an efficient delivery of bioactive molecules e.g. targeting it to inflammatory cells.

**Research Conclusions.** Various bioconjugation techniques have been presented in Chapter 1, out of which the two main pH-sensitive ones are boronic-diol and acetal linkages. Acetals, like boronic-diol linkages, hydrolyse at acidic pH and hence have been used for drug delivery of anticancer drugs. The main advantage the boronic-diol technique has is that the hydrolysis can occur quickly at less acidic pH’s like 5.5 – 6 of the endosome as compared to acetals which have a slower release and require a much more acidic medium of around pH 5 which is found in lysosomes. Another advantage is that the equilibrium constants for boronic-diol conjugates are low, providing easier decomplexation.

Boronic acids can be linked to polymers like HA which contain carboxylic acid and hydroxyl groups by various different functionalisation techniques, some of which have been reviewed in Chapter 1. One of the main reasons for choosing a polymer like HA is its biodegradability in vivo by hyaluronidase enzymes which needs to be retained even after functionalisation. The carboxylic acid group of HA is preferred for
functionalisation since there is only one group per repeating unit, hence allowing for minimum structural changes to the HA.

A new technique for studying the complexation of boronic acids with diols (catechols, flavonoids, sugars, etc.) using ARS as an absorbance reporter has been presented in Chapter 2. In terms of equilibrium constants, the results obtained with this method are comparable to those of fluorescence-based assays. The main advantage of this method is its application to boronic-diol reactions in complex systems, such as in a self-emulsifying system (Tween 20 + quercetin) and during enzymatic reactions (for example: conversion of estradiol to 2-hydroxyestradiol using CYP1A2). Accounting for the diversity of systems the batochromic shift method works for, it can be assumed that it would act as a good reporter for these reactions in a biological environment.

Note: the two examples of enzymatic reactions have a specific relevance: the use of a colorimetric test to follow hydroxylation of cyclodextrin-complexed estradiol by CYP1A2 to 2-hydroxyestradiol could possibly allow to follow tumour development as the 2-hydroxy derivative is non-cancerogenic and tumour-inhibiting because of its rapid monomethylation by COMT.

We have also presented for the first time the demethylation of 3-methoxytyramine by CYP2D6 to dopamine, which is a major drug for Parkinson’s disease. We have shown that CYP2D6 is able to regenerate dopamine from its COMT-derived inactivation product, suggesting that the efficacy of catecholamine-based PD therapy may be influenced by the interplay between these two enzymes and by their polymorphisms.

In Chapter 3 we have presented the synthesis of HA derivatives (-boronic, -dopamine and -veratrylamine) using a simple amine coupling technique. HA-boronic was synthesised to serve as a drug delivery carrier for diol containing bioactive molecules using boronic-diol complexation chemistry while HA-dopamine (and its control HA-veratrylamine) can be used for cell and/or other surface functionalisation (for example: titanium dioxide surfaces) for biomedical devices.

The study of the cytotoxicity of the HA derivatives on fibroblasts and macrophages revealed that HA-boronic was the least toxic, maintaining the highest cell viability even at remarkably high concentrations. All the derivatives showed a lower degradability than their parent polymer, decreasing with increasing degree of derivatisation. In terms of drug delivery carriers, this ensures a prolonged circulation time and recognition by the degrading enzymes. These results suggest the possibility of a rather long circulation
time (stability against degradation) without remarkable toxic effects, and are therefore encouraging in view of the application of HA-boronic as a drug delivery carrier. The equilibrium constants obtained for the complexation with HA-boronic were comparable to those obtained with 3-APBA, ensuring adequate binding of the diols to the carrier, and signs of coil contraction were seen with SLS.

**Future work.** Future work should be focused on studying the uptake of these bioconjugates by cells through receptor mediated endocytosis, followed by the release of the drug and the breakdown of the polymeric carrier *in vitro*. Once a particular drug is selected for delivery, the process of bioconjugation can be optimized for optimal pH of binding and the kinetics for release on reducing pH, followed by the studies of uptake of the bioconjugate by cells and the release of the drug *in vitro* at first and then *in vivo*. Future studies could also involve the synthesis of microgels for drug delivery which would be pH responsive using the boronic-diol chemistry by using a boronic-derivatised polymeric carrier crosslinked to a diol containing drug.

Various diol containing drugs can be delivered using this strategy and it might be useful for studying the conjugation and release of these drugs depending on their application; for example: anti-inflammatory drugs such as catechol, 3- and 4-methylecatechol, 4-tert-butylcatechol, etc. can be delivered to leukocytes where they could be released and possibly reduce inflammation while the HA would be degraded enzymatically. Various anticancer drugs like 2-hydroxyestradiol, 3-(8′(Z), 11′(Z)-pentadecadienyl) catechol, caffeic acid, Cis 3,4-dihydroxycinnamic acid, 3,4-dihydroxybenzaldehyde, etc. can be delivered to tumour cells using the HA carrier where they could possibly induce apoptosis and block cell cycle while the HA could be degraded by the hyaluronidases. Also antihistamines like vitamin C, etc. can be delivered to vascular smooth muscle, glandular cells, endothelium and mast cells where they could prevent histamine release and increase the detoxification of histamine leading to treatment of allergies and/or control of inflammation.