Insights into dynamic covalent chemistry for bioconjugation applications

SHUJIANG WANG
Dynamic covalent chemistry (DCC) is currently exploited in several areas of biomedical applications such as in drug discovery, sensing, molecular separation, catalysis etc. Hydrazone and oxime chemistry have several advantages, such as mild reaction conditions, selectivity, efficiency, and biocompatibility and therefore, have the potential to be for bioconjugation applications. However, these reactions suffer from major drawbacks of slow reaction rate and poor bond stability under physiological conditions. In this regard, the work presented in this thesis focuses on designing novel bioconjugation reactions amenable under physiological conditions with tunable reaction kinetics and conjugation stability.

The first part of the thesis presents different strategies of dynamic covalent reactions utilized for biomedical applications. In the next part, a detailed study related to the mechanism and catalysis of oxime chemistry was investigated in the presence of various catalysts. Aniline, carboxylate and saline were selective as target catalysts and their reaction kinetics were compared under physiological conditions (Paper I and II). Then we attempted to explore the potential of those chemistries in fabricating 3D hydrogel scaffolds for regenerative medicine application. A novel mild and regioselective method was devised to introduce an aldehyde moiety onto glycosaminoglycans structure. This involved the introduction of amino glycerol to glycosaminoglycans, followed by regioselective oxidation of tailed flexible diol without affecting the C2-C3 diol groups on the disaccharide repeating unit. The oxidation rate of the tailed flexible diol was 4-times faster than that of C2-C3 diol groups of native glycosaminoglycan. This strategy preserves the structural integrity of the glycosaminoglycans and provides a functional aldehyde moiety (Paper III). Further, different types of hydrazones were designed and their hydrolytic stability under acidic condition was carefully evaluated. The hydrazone linkage with the highest hydrolytic stability was utilized in the preparation of extracellular matrix hydrogel for delivery of bone morphogenetic proteins 2 in bone regeneration (Paper IV) and studied for controlled release of the growth factor (Paper III).

In summary, this thesis presents a selection of strategies for designing bioconjugation chemistries that possess tunable stability and reaction kinetics under physiological conditions. These chemistries are powerful tools for conjugation of biomolecules for the biomedical applications.

Keywords: dynamic covalent chemistry, reaction mechanism, hydrogel, bioconjugation, catalysis

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To my beloved family

献给我的家人
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Publications not included in this thesis


My contributions to the papers in this thesis

I. I contributed to the design of the study, performed all the experiments except for cell surface labelling. I participated in the discussion of the results and wrote the manuscript.

II. I contributed to the design of the study, performed all the kinetics study, participated in the discussion of the results and wrote part of the manuscript.

III. I contributed to the design of the study, performed all the experiments except ELISA, participated in the discussion of the results and wrote main part of the manuscript.

IV. I contributed to the synthesis and characterization of HA derivative and HA hydrogel, performed kinetics study, participated in the discussion of results and wrote part of the manuscript.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>DCC</td>
<td>Dynamic covalent chemistry</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>CD</td>
<td>Cyclodextrin</td>
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<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic proteins</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>HOBt</td>
<td>N-hydroxybenzotrizole</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>DOX</td>
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<td>Hyaluronic acid</td>
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<tr>
<td>HP</td>
<td>Heparin</td>
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<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>dPBS</td>
<td>Deuterated phosphate buffer saline</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>Attenuated total reflectance Fourier transform infrared</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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Scope of thesis

Designing novel covalent coupling chemistries that can be performed under physiological conditions without using any toxic reagents or yielding toxic by-products are essential for bioconjugation applications. In addition, these chemistries should have tunable reaction kinetics (optimal reaction time) and yield stable products. However, for certain applications, such as drug delivery, a linkage with tunable stability is required. Dynamic covalent chemistry (DCC) is one such promising candidate that has been extensively used for biological and biomedical applications. By adjusting reaction conditions, reactant structures or by utilising different catalysts, one can tune these chemistries for diverse applications.

The work presented in this thesis is focused on hydrazone and oxime chemistries, which can be performed efficiently and selectively under mild conditions and at physiological pH. Oxime products are known to exhibit superior hydrolytic stability as compared to the hydrazone products; however, it has limited application in bioconjugation due to slow reaction rate under physiological conditions, especially with keto substrates. Moreover, this chemistry requires a high substrate concentration to achieve a reasonable reaction rate, which is difficult for many reactions involving biomolecules. To optimise oxime reaction for bioconjugation applications, the mechanism and catalysis of oxime formations with aldehyde and ketone as the substrate were investigated under physiological conditions. Different concentrations of aniline, carboxylate and saline were used to promote oxime formation under our experimental conditions, and the reactions were monitored by $^1$H NMR, UV-vis spectroscopy and analysed by computational analysis (in Paper I and II).

The dynamic covalent coupling reactions such as hydrazone and oxime reactions have been extensively used for several bioconjugation applications. Since these chemistries are mild and can be performed in the presence of other functional groups (bioorthogonal), they have been employed to develop extracellular matrix mimetic materials and hydrogel scaffolds.

Hydrogels are attractive materials that are extensively hydrated (nearly 98% water) and have a wide range of application such as drug or nanocarrier depot, a scaffold for tissue regeneration, 3D cell culture and so on. One of the major limitations of hydrogels fabricated with this oxime and hydrazone crosslinking chemistries is their excessive and uncontrolled swelling. This phenomenon arises due to the reversible nature of the crosslinks. To design
an extracellular matrix mimic hydrogel with good hydrolytic stability, controlled swelling and bioactivity, we investigated various hydrazone linkages to resolve this problem.

First, a mild and efficient strategy was designed to conjugate aldehyde functional group to glycosaminoglycan regioselectively (Paper III). Regioselectivity was investigated by exploring periodate oxidation kinetics with amino-glycerol modified and native (unmodified) glycosaminoglycan substrates. Then, three different types of hydrazone were designed and utilised to prepare extracellular matrix mimic hydrogel (Paper IV). Those hydrogels were characterised by hydrolytic degradation, enzymatic degradation and rheology studies. The most suitable hydrogel was used for bone tissue engineering applications where it was used as a carrier of bone morphogenetic proteins 2, a sensitive growth factor necessary for bone regeneration (Paper IV). We succeeded in using this system for the controlled release of other growth factors (Paper III).

In summary, this thesis presents a selection of strategies for designing bioconjugation chemistries to be performed under physiological conditions. Those chemistries have tunable stability and reaction kinetics, which enables their application in the biological and biomedical field.
1 Introduction

Biomolecules exist in all living organisms and are essential for specific biological processes. In order to understand the complex biological processes, new chemistries are developed with special emphasis on the design, synthesis, and characterization of the product, such that these chemistries do not have any detrimental effect on the biomolecule of interest.¹ Natural biomolecules often exist as complexes and require advanced isolation techniques for purification. On the other hand, numerous natural biomolecules are unstable and difficult to isolate, thus strategies to modify biomolecules to increase their stability and detectability are crucial for biological and biomedical research. A promising approach is to utilise bioconjugation techniques, that refers to the covalent or non-covalent linking of biomolecules to other substrates.² It can be applied to various macromolecules such as polysaccharides, nucleic acids, proteins, peptides, lipids as well as small molecules.

Ideal bioconjugation strategy should be non-toxic, chemoselective, efficient even with a low substrate concentration (sub-micromolar) and stable under physiological conditions. These reactions should be bioorthogonal such that it should not interfere with the surrounding biological environment or biological processes.³ Over the past several decades, non-covalent interactions have been used for conjugating different biomolecules for labelling studies, developing drug delivery systems etc.² Those interactions include electrostatic interaction, metal-mediated coordination, hydrophobic interactions, and hydrogen bonding etc. Although these interactions usually possess rapid reaction kinetics, they lack stability or chemospecificity for bioconjugation applications as compared to covalent coupling strategies., Therefore, many of the current bioconjugation strategies are concentrated on the covalent coupling reactions, which leading to a strong and stable linkage.²

Conventional covalent bioconjugation strategies utilise the native functional groups of target biomolecules resulting in the loss of their biological function.³, ⁴ Moreover, the covalent bioconjugation reaction is generally slower than non-covalent bioconjugation, thus organic catalysts are commonly used to promote such conjugation reactions. Recently, a growing research interest is focused on the design of novel bioconjugations with high site-specificity, efficiency, extensive environmental tolerance and negligible interfere with the biological functionality of biomolecule.³
### 1.1 Dynamic covalent chemistry

Dynamic covalent chemistry (DCC) involves the reversible bond formation and cleavage of covalent bonds under thermodynamic control. The tunability of the reaction kinetics and bond stability is the main advantage of dynamic covalent chemistry as compared to the other bioconjugation methods. Dynamic covalent reactions can be tuned by modifying reaction conditions (e.g. solvent, temperature, pH, and charge), adjusting the ratio of the substrate and by modifying substrate structure etc.

Among such dynamic reactions, imine/hydrazine/oxime reactions, aldol reaction, reversible Diels-Alder reaction, thiol-Michael reaction, transesterification reaction, boronic acid condensation, disulphide exchange reactions are of particular interests. These reactions can be formed selectively and specifically under mild conditions (Scheme 1.1) and are therefore powerful tools for bioconjugation applications.

**Scheme 1.1** Common dynamic covalent reactions for bioconjugation.
1.1.1 Hydrazone and oxime

Hydrazone and oxime are the most classic and widely used dynamic covalent reactions that can trace back to 1880s.\(^6,8\) The classical reaction mechanism involves the general acid catalysed attacking of \(\alpha\)-nucleophile (hydrazide or aminooxy) to carbonyl carbon, followed by water elimination to form the oxime product (Scheme 1.2).\(^9\) Rate limiting step of hydrazone and oxime formation varies at different pH. The rate limiting step is nucleophilic attacking when pH value is below 3, while dehydration is the rate-limiting step between pH 3-7.\(^7,9,10\)

\[
\begin{align*}
\text{RCH} &= H^+ + \text{H}_2\text{O} \quad \text{Hydrazide} \\
\text{RNH} &= \text{RNHOH} \quad \text{Oxime}
\end{align*}
\]

These chemistries have several characteristics that make them extremely suitable for biomolecule conjugations. First, the reaction could be carried out selectively and efficiently under mild conditions in an aqueous medium, which is compulsory for many biological processes. Secondly, the reaction occurs between carbonyl and \(\alpha\)-nucleophile with water as the only by-product, thus the reactions are compatible with many biomolecules. Thirdly, equilibrium of the reversible reactions is under thermodynamic control, and the reaction kinetics and product stability could be easily adjusted by modifying reaction conditions or reactant structure. Although these chemistries are powerful tools for biomolecule linkage, they have limitations for the bioconjugation application, such as instability in aqueous medium, slow reaction kinetics at physiological pH and poor reactivity with ketone substrate.

Reaction kinetics of hydrazone and oxime are affected by several factors. The reactions have poor reactivity under physiological conditions, while at slightly acid pH (around pH 4.5) or in the presence of nucleophilic catalysts the reaction rate enhanced significantly. Aniline is the most commonly used nucleophilic catalyst that can catalyse hydrazone and oxime formation under physiological condition. Dawson and coworkers explored aniline catalysed oxime reaction for peptide conjugation at pH 4.5 and pH 7.0 and demonstrated that the conjugation reaction is much more effective at pH 4.5.\(^\text{11}\) They also demonstrated that aniline catalysed oxime reaction is capable of cell surface labelling with a fluorescent tag having aminooxy functional group.\(^\text{12}\) In order to improve the solubility and catalytic efficiency, a variety of aniline derivatives were designed, such as p-methoxyaniline,\(^\text{11}\) anthranilic acid,\(^\text{13}\)
2,4-dimethoxyaniline, methoxyanthranlic acid and phenyldiamine. In this thesis, we studied the mechanism and catalysis of oxime chemistry under physiological conditions and utilized carboxylate and saline to catalyse oxime formation at physiological pH (Paper I and II).

Substrate structure also influences the reaction kinetics. In general, hydrazide exhibits higher reactivity than aminooxy, and aldehydes are more reactive than comparable ketone. Aromatic aldehydes usually have lower reactivity than aliphatic aldehydes, but reactivity improved significantly by introducing electron withdrawing substituents, such as ortho-boronic acid and nearby acid or basic groups. The reactivity of hydrazide could be enhanced by introducing nearby acid group as well. Jianmin Gao and co-worker designed ortho-boronic acid substituted aromatic aldehyde or ketone that enables fast conjugation to fluorophore- labelled semicarbazide, and this reaction is capable of detecting the bacterial pathogen in blood serum.

Stability of hydrazone and oxime is sensitive to the reaction environment. They undergo rapid hydrolysis under acidic conditions while stable at physiological pH. In additions, hydrazone cleaves rapidly in specific in vivo environment. For example, aroylhydrazone exhibited fast hydrolysis in plasma; proteinogenic amino acids that exist in serum could accelerate the hydrolysis of hydrazone.

Substrate structure also affects the linkage stability. Hydrazone obtained from ketone substrate possess enhanced hydrolytic stability than the corresponding aldehyde. The aromatic aldehyde also forms a more stable oxime than aliphatic aldehydes/ketones. Oxime normally exhibits superior hydrolytic stability as compared to hydrazone attributed to the α-effect of the aminooxy oxygen. We utilised this α-effect to design a hydrazone linkage with extraordinary hydrolytic stability and controlled enzymatic (Paper IV).

1.1.2 Other dynamic covalent chemistries: advantage and disadvantage

1.1.2.1 Imine chemistry

Imine is formed by the condensation reaction between carbonyl and amino functional groups. The reaction is reversible and the equilibrium of the reaction is towards the imine formation in the presence of catalysts or dehydrating reagents. Although imine exists in various natural molecules, it is not commonly used as conjugation chemistry for biomedical applications due to several reasons. First, imine products are generally unstable under aqueous conditions and undergo rapid hydrolytic cleavage. Secondly, imine formation is slow under physiological conditions and requires catalysts to promote this reaction at a reasonable rate. In addition, imine could react with other molecules and lead to the formation of unwanted by-products.
1.1.2.2 Reversible Diels-Alder reaction

Diels-Alder reaction is a powerful tool for synthesis of the six-membered cyclic structure by the reaction of a conjugated diene and a substituted alkene. This reaction possesses high atom efficiency and good regio- and stereoselectivity. Diels-Alder reaction is exothermic and normally the product is stable at room temperature. This makes the reaction most suitable for bioconjugation applications. However, when the reaction is carried out with electron rich dienes and electron-poor dienophile reactant, the reversible Diels-Alder could be performed at room temperature.6 Retro Diels-Alder reaction with furan and maleimide substrate is reversible around 40 °C.23 The retro Diels-Alder reaction between anthracene and tetracyanoethylene can be performed at room temperature.24

The equilibrium of reversible Diels-Alder reactions could be influenced by temperature, solvent, and substrate concentration, but it is not sensitive to water, which is a big advantage for bioconjugation application.25 The reaction kinetics and stereo selectivity of reversible Diels-Alder reaction can be adjusted by modifying substituents of the substrates. By modifying substrate structure, the reversibility of Diels-Alder reaction changed significantly, from irreversible largely exergonic reactions to mildly exergonic reversible reactions or unfavourable endergonic reactions.25 Diels-Alder could be used to generate dynamic covalent libraries,26 oligosaccharides conjugation,27 hydrogel preparation28 and so on. The major disadvantage of this reaction is the requirement of specific substrate structure.

1.1.2.3 Aldol reaction

Aldol reaction is a widely used tool to form a C-C bond in organic synthesis. This reaction involves the reaction of two carbonyls to form β-hydroxy carbonyl compounds, and the hydroxyl and aldehyde functional group in the product can be used for further modification.4,29 It is known that the aldol reaction is catalysed by acid and base. However, these catalysts are not bio-compatible; meanwhile, they could lead to the unwanted dehydration by-product formations. In addition, these reactions lack chemo-, regio-, and stereoselectivity.30 Therefore, chiral inductor, ligands and catalysts are used to promote asymmetric aldol reaction.30 Enzymes, such as aldolases, are the most used catalyst for aldol reactions under physiological conditions.3, 31 Antibodies,32 and amino acid 33 are utilized to accelerate aldol reaction as well.

1.1.2.4 Transesterification

Transesterification is a classic and extensively studied bioconjugation reaction that involves the exchange of alkoxy moiety between an ester and alcohol.34 This reaction occurs simply by mixing the two substrates and could be catalysed by acid, base, and enzyme.34 Transesterification is useful
when the parental carboxylic acid is difficult to isolate. The reaction has been widely used in academic research and industrial manufacture.

1.1.2.5 Disulphide exchange
The disulphide exchange reaction is one of the oldest and most widely used dynamic covalent chemistries. It involves thiol exchange between an active disulphide and a deprotonated thiol. The reaction is highly selective to thiols and exhibits good resistance to hydrolysis. Though the reaction can be carried out over a broad range of pH, it is more favourable under basic condition. Normally the reaction is performed at pH 7-9 to ensure sufficient amount of thiolate formation.

Disulphide exchange is an essential reaction for all living species since it is crucial for the protein folding process. This reaction is an important conjugation method for biomaterials modification. Pyridyl disulphide derivatives are the most commonly used reagents for disulphide exchange and the by-product pyridine-2-thione is UV-Vis detectable, therefore the progress of this reaction could monitor by UV-Vis spectroscopy.

However, disulphide bond is sensitive to reduction environment. For instance, the disulphide bond can be reduced by glutathione in vivo. Therefore as a bioconjugation reaction, disulphide exchange is more suitable for in vitro studies.

1.1.2.6 Thiol-Michael addition
Thiol-Michael addition is another important bioconjugation method. It refers to the 1,4-addition of thiol to α,β-unsaturated carbonyl to form a C-S bond. The rate-limiting step of the thiol-Michael addition reaction is the nucleophilic attack of a thiolate anion on the electron-deficient vinyl. This reaction is a power tool to conjugate a wide-range of thiol containing molecules like proteins, peptides, and drugs.

Thiol group in cysteine is the most commonly used target for this conjugation. Another common reactant is maleimide, which undergoes thiol addition between pH 6.5 and 7.5. The resultant adduct normally takes long reaction time to reach equilibrium. Recently, a tunable thiol-maleimide reaction with the N-ethylmaleimide substrate has been reported. This reaction has been applied in controlled release, and degradation kinetics of thiol-maleimide adducts can be modified by adjusting the Michael donor’s reactivity.

1.1.2.7 Boronic acid condensation
Boronic acid condensation is one of the most popular dynamic covalent chemistries. Boronic acid can underdo self-condense or combine with diols to form a cyclic product. The reaction between boronic acid and diol is fast and the resultant boronic ester bond is stable, while it is also reversible under
certain conditions. This reaction has been used to develop molecular sensors, prepare hydrogels, synthesise nanomaterials, cell culture etc.

Although the number of dynamic covalent reactions is growing continuously, there are still limited variety of DCC that are suitable for bioconjugation applications for several reasons, such as harsh reaction conditions, slow kinetics, and limited aqueous solubility etc. Attributed to the discovery of novel biocompatible dynamic covalent reactions and catalysts in last few decades, DCC has been extensively used in several bio-related areas, such as drug delivery, hydrogel synthesis, labelling, gene transfection and so on.

1.2 Dynamic covalent chemistries in hydrogel preparation

Hydrogel represents a three-dimensional hydrophilic polymer network formed by chemical cross-linking or physical interaction. The physical interactions including chain entanglements, electrostatic interaction, hydrogen bonds, van der Waals interactions or crystallites. The physical cross-linking hydrogels possesses some superior properties, such as shear thinning, self-healing and dynamic crosslink exchange, which makes it extremely suitable for designing 3D printing hydrogel and self-healing hydrogel. This type of hydrogels also avoids using toxic crosslinker or initiators, which allows the application in cell encapsulation. Limitation of most physical cross-linking hydrogels includes uncontrolled degradation and weak mechanical strength.

On the other hand, chemically cross-linked hydrogel usually has higher stability and mechanical strength as compared to the physically cross-linked hydrogels. Chemically cross-linked hydrogels could be prepared by using multi-functional crosslinker, such polyethene glycol (PEG), glutaraldehyde etc. However, the potential intramolecular cross-linking leading to the formation of polymer loops, which could reduce the mechanical strength and network stability of hydrogels. In addition, several popular small molecule linkers like glutaraldehyde are toxic and require further purification after hydrogel formation. An alternative method to synthesise chemical cross-linking hydrogels is to introduce bioorthogonal function groups to different polymer chains and gelling occurs after mixing the two different types of multifunctional polymer chains. A variety of dynamic covalent chemistries has been used to synthesise hydrogel for biomedical applications.

Hydrazone and oxime crosslinking hydrogel have been used as extracellular matrix (ECM) mimic scaffold for tissue regeneration attributed to its efficiency, selectivity and mild reaction conditions, but excess and uncontrolled swelling in vivo is a major challenge for biomedical application. In this thesis, a novel hydrazone was designed and used to tailor a hyaluronic
acid hydrogel as BMP-2 carrier, which exhibited extraordinary hydrolytic and enzymatic stability, good mechanical strength and low swelling (Paper IV).

Imine reaction has been extensively used for hydrogel preparation. An injectable hydrogel based on aldehyde modified dextran and cyclodextrin (CD)-functionalized polyaspartamide with PEG pendants (PG) for hydrophobic drug delivery has been reported. PEG helps to increase the degree of CD substitution on polyaspartamide, while the CD is used to encapsulate hydrophobic drug.

Boronic acid containing hydrogels have some specific properties such as glucose-responsivity and self-healing. These hydrogels have applications in sensor design, controlled drug release, HIV-Barrier and 3D cell culture.

Diels-Alder reaction has high selectivity and high atom efficiency, and furan-based Diels-Alder reactions have been widely used in biocompatible materials preparations. However, some functional groups of this reaction have limited aqueous solubility, and normally the retro Diels-Alder reaction is difficult at room temperature. Yong Mei Chen and co-workers designed a self-healing dextran hydrogel using fulvene-modified dextran and dichloromaleic-acid-modified poly(ethylene glycol). This hydrogel can be made under physiological conditions and has good potential for biomedical applications.

The thiol-disulphide exchange reaction has been utilized to prepare bisphosphonate-modified hyaluronic acid under a more effective and controlled way, and this HA-derivative was used to prepare dually cross-linked self-healing hydrogel for bone regeneration.

Thiol-Michael addition is compatible with the aqueous environment and could be carried out at physiological pH at room temperature. A tunable dextran-based hydrogel for cell encapsulation was prepared by Michael addition. By adjusting the pH of reaction system from 7.0 to 7.8, the gelling kinetics, stiffness of hydrogel, swelling ratio and hydrogel surface morphology can be easily modified.

1.3 Hydrogels in regenerative medicine

Regenerative medicine focuses on replacement, repair or restoration of damaged organs/tissues due to disease, trauma, ageing or injury. Hydrogels have strong hydration capability and interconnected porous structure that allow the diffusion of oxygen, nutrition, bioactive molecules. Therefore, hydrogels have huge potential for several biomedical applications, such as drug delivery, encapsulation, implant, scaffold, cell delivery, barrier, bioreactor, space filler, entrapment, glue, and so on.

Hydrogels can be prepared from synthetic polymers or naturally derived biopolymer. Synthetic polymer-based hydrogels possess well-controlled
structure and good mechanical strength, but lacks biological functions and biocompatibility. Compared to synthetic polymers, natural-origin polymers like peptide, protein, nucleic acid and carbohydrates exhibit excellent biocompatibility and biological activity, which is important for biological and biomedical applications. Numerous nature polymers have been used to prepare hydrogels for regenerative medicine application.

1.3.1 Hyaluronic acid

![Figure 1.1 Structure of hyaluronic acid](image)

Hyaluronic acid (HA) is one of the major components of extracellular matrix that consists of D-glucuronic acid and N-acetyl-D glucosamine. It is the only non-sulphated glycosaminoglycan (GAG) and is abundant in extracellular matrix, skin, lungs and intestine. HA is synthesised by a class of integral membrane proteins named hyaluronan synapses in vivo, and the molecular weight can reach up to 20,000 kDa. It can be degraded by an enzyme named hyaluronidase that exists in the extracellular matrix causing hydrolysis under acidic or basic conditions and thermal decomposition. HA plays an important role in wound healing, cell proliferation, lubrication and so on. It is notable that the biological function of HA is influenced by its molecular weight. For example, the native high molecule weight HA plays a role in hydration and maintaining cell integrity, while the degraded low molecule HA exhibits pro-inflammatory properties.

Due to the short in vivo half-life of HA, chemical modification is normally performed to increase its stability and functionality. Most of those modifications are performed on the carboxylic acid moiety or the C-6 hydroxyl group. It is also possible to modify the C-6 hydroxyl group by etherification using epoxides, divinyl sulfone, esterification using acid anhydride, or form hemiacetal analogues using glutaraldehyde. Since native HA is almost insoluble in organic solvent, and undergoes hydrolysis in acidic and basic conditions, the modification is usually performed under a mild condition in aqueous medium or polar organic solvent like DMSO.

HA hydrogels have been extensively used for regenerative medicine. In this thesis, hydrazone cross-linked HA hydrogels were synthesized. This hydrogel exhibited extraordinary resistance to hydrolysis and was used as sufficient carrier to deliver bone morphology protein-2 (BMP-2) for bone
regeneration (Paper IV). These hydrogels have also been used for controlled release of therapeutic proteins (Paper III).

1.3.2 Chondroitin sulphate

![Structure of chondroitin sulphate](image)

Chondroitin sulphate (CS) is a hydrophilic glycosaminoglycan that consists of alternating sulphated N-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid (GlcA). CS can be chemically modified on the carboxylic acid group of D-glucuronic acid or the hydroxyl group. As one of the major components of cartilage, CS plays an essential role in the water retention, which is important for pressure resistance. It exhibited binding ability of certain growth factors and has been used in cartilage regeneration, drug delivery and cell encapsulation.

1.3.3 Heparin

![Structure of heparin](image)

Heparin (HP) is a heterogeneous GAG that consisting of α-L-iduronic acid, β-D-glucuronic acid, and α-D-glucosamine residues. HP is abundant in the liver, and the highly negatively charged structure of HP allows its electrostatic binding to proteins and growth factors. Heparin and heparin sulphate have specific binding sites of some proteins such as FGF-2 and could stabilize those proteins within the ECM. HP also has anticoagulant properties, which can be used to prepare the anticoagulant coating. HP hydrogel has been utilized for the tissue regeneration, cell encapsulation and controlled delivery of bioactive molecules.
2 Result and discussion

2.1 Mechanism and catalysis of oxime chemistry

Oxime and hydrazone chemistries are the most successful bioorthogonal chemistries that have a wide range application in various fields. However, oxime and hydrazone have the slow reaction rate with a low substrates concentration under physiological pH, which is a major limitation for the bioconjugation applications.\textsuperscript{43} In the last decade, oxime and hydrazone became one of the most versatile reactions attributed to the discovery of aniline as nucleophilic catalysts.\textsuperscript{58} Although aniline exhibits good catalytic efficiency under the acidic environment (pH 4.5), it has several limitations including low efficiency under physiological conditions, not efficient with ketone substrates, genotoxicity,\textsuperscript{59} low solubility in aqueous medium etc. In order to design a new biocompatible catalyst that is efficient at neutral pH with both aldehyde and ketone substrates, we first investigated the mechanism and catalysis of oxime reactions at neutral pH.

2.1.1 Aniline and carboxylate catalysis of oxime ligation

2.1.1.1 Pseudo-first-order oxime ligation kinetics

Figure 2.1 Representative $^1$H NMR of oxime reaction between acetone and methoxymine.
To determine the catalysis of selected catalyst in oxime reaction, a series of pseudo-first-order reactions kinetics studies were performed in 10 mM of deuterated phosphate buffer saline (dPBS, pD 7.4) at 25 °C. 10 mM water-soluble aldehydes and ketones were used as the model carbonyls and 1 mM of water-soluble methoxyamine (NH₂OCH₃) was used as a model nucleophile. The conversion of the reaction was calculated from the ratio of the methyl peak in free methoxyamine at 3.57 ppm and at 3.82 ppm of oxime product in ¹H NMR (Figure 2.1).²¹

2.1.1.2 Aniline catalyzed oxime mechanism

Scheme 2.1 Mechanism of aniline catalysed hydrazone/oxime reaction.

Aniline catalyzed oxime and hydrazone reactions through the transimination mechanism, which involves the nucleophilic addition of aniline to the carbonyl, followed by dehydration to form the imine intermediates. The α-nucleophiles further attacks the imine intermediate forming the desired product after water elimination (Scheme 2.1).

Table 2.1. Pseudo-first-order kinetics for oxime formation with acetone (Ac) and 4-hydroxybenzaldehyde (4-HB) substrates. AO = methoxyamine; Ac = Acetone; k₁ = pseudo first-order rate constant; r = relative rate; ND = not determined.

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</tr>
</tbody>
</table>

We first explored aniline catalysis in aldehyde based oxime reaction. Here acetaldehyde was used as the model aliphatic aldehyde, and 4-hydroxybenzaldehyde was used as the model aromatic aldehyde. The oxime reaction with acetaldehyde substrate was extremely fast and reaction kinetics could not be monitored by ¹H NMR analysis. When 4-hydroxybenzaldehyde was used as the substrate, the reaction rate was slow without any catalyst, while the reaction rate accelerated significantly in the presence of 100 mM aniline (Table 2.1).
Scheme 2.2 Pathway [I] represents reaction mechanism when the concentration of aminooxy derivative is higher than acetone; Pathway [II] represents reaction mechanism involving aniline when the concentration of aminooxy derivative is lower than acetone. Since the ketone-based reaction is more challenging as compared to the aldehyde-based reaction, we investigated the catalysis of oxime reaction in acetone-based reaction. As shown in Table 2.1, aniline enhances the oxime formation rate by ~1.7-folds when the concentration of acetone is higher than methoxyamine. We believe when ketone is used as the substrate, proton transfer occurs between the aminooxy nitrogen atom (intermediate 7) and the neighbouring ether oxygen (intermediate 8) due to the α-effect of this oxygen, which restricts the formation of key intermediate 9 that can undergo dehydration to form desired product 10 (pathway I in Scheme 2.2). In the case of aniline, similar proton transfer occurs between the intermediate 14 (due to α-effect) and 16, thus the formation of key intermediate 15 (pathway II in Scheme 2.2) is restricted. Interestingly, aniline did not exhibit any catalysis when 10 folder excess of aminooxy was used for oxime reaction (Figure 2.1).14 We believe this is attributed to the competing reaction of aniline and methoxyamine since they have similar pKa value ~4.6.60,61

2.1.1.3 Acetate activated oxime mechanism

Anilinium has a pKₐ value around 4.6,60 therefore its nucleophilicity and protonation ability plays a role in the catalysis simultaneously. In order to clarify the contribution of each part, we explored the catalysis of acetic acid, a non-nucleophilic catalyst that has similar pKₐ (~4.7) as anilinium.62 As shown in Table 2.1, acetate can activate aldehyde-based oxime reaction.100
mM sodium acetate accelerates the 4-hydroxybenzaldehyde based reaction by ~1.5-folds. This acceleration is further enhanced to ~6.4-folds by increasing the concentration of sodium acetate to 1 M. Interestingly, although pyridinium has a higher pK_a of 5.5 and possesses higher proton donating potential at physiological pH as compared to anilinium and acetic acid, it did not stimulate any activation of oxime reaction. This indicates that catalysis of carboxylate in oxime reaction is not only attributed to acid catalysis, and the mechanism of acetate catalysis is not simply following conventional imine formation mechanism.

![Figure 2.2](image)

**Figure 2.2.** Comparative pseudo first-order reaction kinetics at different substrate ratio using aniline or acetate as catalyst.

![Scheme 2.3](image)

**Scheme 2.3.** a) The proposed mechanism of acetate activated oxime reaction of ketones; b) Proposed mechanism of anilinium acetate promoted oxime reaction of ketones.

Interestingly, the catalysis of sodium acetate in acetone-based oxime formation can overcome the α-effect. The rate of oxime formation increased by
~1.5-fold in the presence of 100 mM sodium acetate, and increases to ~4.3-fold in the presence of 1 M sodium acetate (Figure 2.2 and Table 2.1). In addition, this catalysis is independent of the ratio between acetone and methoxyamine. We believe acetate catalysis ketone-based oxime formation through a mechanism similar to Passerini reaction. First, carboxylate forms the hydrogen bond (H-bonds) with acetone, and the resultant intermediate 17 helps the nucleophilic attacking of aminoxy (Scheme 2.7) to give the key intermediate 8. Then the intermediate 8 forms a neutral species with acetate through H-bond, and this H-bond assists in the protonation of hydroxyl oxygen (intermediate 19), which undergoes dehydration to form the oxime (Scheme 2.3).

![Figure 2.3 ATR-FTIR spectrum of acetone (black line); acetone: acetic acid=1:1 v/v (red line) and acetone:water = 1:1 v/v (blue line).](image)

In order to confirm the H-bonds between acetone and acetic acid, we performed attenuated total reflectance Fourier transform infrared (ATR-FTIR) analysis of pure acetone, acetone/acetic acid mixture (1/1, v/v) and acetone/water mixture (1/1, v/v). According to previously published research, the C=O stretching frequency display a red shift from 1711 cm\(^{-1}\) in pure acetone to 1698 cm\(^{-1}\) in the acetone-water mixture and 1701 cm\(^{-1}\) in the acetone-acetic acid mixture, which indicates the strong H-bonds between the ketone and carboxylic acid (Figure 2.3).\(^{63}\) We also observed a strong blue shift of ~30 cm\(^{-1}\) from 1390 cm\(^{-1}\) in the acetone-acetic acid mixture to 1369 cm\(^{-1}\) in the acetone-water mixture (Figure 2.3), which indicates the Van der Waals interactions between acetone carbonyl carbon and carboxylic acid carbonyl oxygen that induced the formation of intermediate 17 (Scheme 2.3a).\(^{64}\)
2.1.1.4 Anilinium carboxylate catalyzed oxime mechanism

Inspired by the catalytic efficiency of anthranilic acid, an aniline derivative that contains ortho-carboxylic acid, we investigated the capability of acetate activation in aniline catalysed oxime reaction with acetone substrate. As shown in Figure 2.4a, 100 mM acetate accelerates oxime formation rate by~1.5 fold, and it increased to ~3.5 fold when 100 mM aniline was combined with 100 mM acetate. This rate enhancement increased to ~11 fold by increasing acetate concentration to 1M while keeping aniline concentration at 100 mM. We believe the mechanism of this efficient activation is analogous to the acetate mechanism (Scheme 2.3b), where H-bonds between intermediate 14 and acetate helped the formation of key intermediate 21 that gave oxime product after anilinium elimination.

![Figure 2.4](image.png)

**Figure 2.4** Comparative pseudo-first-order reaction kinetics of oxime formation with 10 mM acetone/ 1 mM aminooxy substrate using different catalysts.

Promoted by the catalytic capability of acetate in acetone-based oxime reaction, we then studied the catalytic capability of other carboxylate-containing reagents that are commonly used in biological and biomedical research, such as sodium trifluoroacetate (TFA) and sodium citrate. 100 mM TFA, anilinium TFA, citrate and anilinium citrate enhanced the oxime formation rate by~2.0-folds, ~2.4-folds, ~1.4-folds and ~3.8-folds, respectively (Figure 2.4). Since 100 mM citrate and 100 mM acetate induced the same rate enhancement, it proved that for each acetone molecule a single carboxylate was required for activation of oxime reaction (Scheme 2.3a and 2.3b).

Interestingly, when the citrate concentration increased to 1 M, we observed a rate increased by ~6.1-folds. The most efficient catalyst is the combination of 1 M citrate and 100 mM aniline, which led to a rate increase by ~14.3-folds (Figure 2.4).
To investigate the versatility of 1 M citrate /100 mM aniline combination in ketone-based oxime formation, 4 different ketones were utilized to perform pseudo-first-order oxime formation kinetics analysis. 4-hydroxybutanone and 2-butanone based reaction exhibited ~14-folds rate increase, while dihydroxyacetone based reaction exhibited ~30-folds rate increase (Figure 2.5). This difference can be attributed to differences in water solubility of different keto.

For the purpose of comparing the catalytic efficiency of anilinium carboxylate with published anthranilic acid, 40 mM of different anilinium carboxylates were utilized to catalyze acetone-based oxime reaction. As shown in Figure 2.6, anthranilic acid catalyzed reaction exhibited similar rate increase as compare to anilinium TFA, anilinium acetate and anilinium citrate (Figure 2.6). We did not explore the reaction kinetics with higher catalysts concentration due to the limited aqueous solubility of anthranilic acid.
Figure 2.6 Comparative pseudo first-order reaction kinetics of 10 mM acetone/1 mM aminoxoy using 40 mM different catalysts in dPBS containing 7% deuterated methanol at pD 7.4. The reaction was monitored by $^1$H NMR.

2.1.1.5 Oxime ligation on cellular surface

Biocompatibility is essential for the catalyst for bioconjugation applications. Although aniline is usually defined as biocompatibility; it exhibited genotoxicity. Limited aqueous solubility is another limitation of aniline in bioconjugation applications. In contrary, acetate has a good aqueous solubility and has been extensively used as a buffer for biological studies.

Figure 2.7 Fluorescence microscopy images of periodate-treated CHO cells subjected to oxime ligation with aminoxoy-biotin in the (A) absence of catalysts; (B) in the presence of aniline; (C) acetate. The nucleus is stained with DAPI and ligation reaction is visualized using FITC labelled avidin.

In order to evaluate the biocompatibility of acetate as catalysts for bioconjugation application, we performed oxime labelling on live Chinese hamster ovarian (CHO) cells surface at pH 7.4 following modified published protocol. Oxime linkage was observed on the cell surface in the presence of 10 mM acetate and 10 mM aniline; on the contrary, cell surface labelling was not observed in the absence of any catalyst.
2.1.2 Saline catalysis of oxime ligation

Although aniline and carboxylate accelerated oxime formation at neutral pH, it is noteworthy that there reactions are always performed in buffers. In addition, NaCl salt that exists in buffers has been reported to catalyse reactions that involve charged intermediate formation, such as Diels–Alder reaction and Michael addition. Since oxime formation involves positive charged hemiaminal intermediate formation as well, we decided to explore the role of saline in oxime reaction.

2.1.2.1 Pseudo-first-order oxime reaction kinetics

![Figure 2.8](image)

Figure 2.8 Representative $^1$H NMR of oxime formation using a) 4-hydroxy benzaldehyde and b) acetone as substrate. c) Comparative pseudo-first-order oxime reaction kinetics of methoxyamine and acetone/4-hydroxybenzaldehyde using different concentrations of NaCl in deuterated phosphate buffer, analyzed by $^1$H NMR. d) Comparative pseudo-first-order oxime reaction kinetics of methoxyamine and 4-nitrobenzaldehyde in PB containing 10% N, N-dimethylformamide, analyzed by UV-Vis spectrometry.

Pseudo-first-order reaction kinetics of small molecule based oxime formation was monitored by $^1$H NMR in 10 mM deuterated phosphate buffer (dPB) with different salt concentration. The reactions were carried using 1 mM carbonyl and 30 mM of methoxyamine, and 4-hydroxy benzaldehyde (Figure 2.8a) and acetone (Figure 2.8b) were used as model carbonyls.

We observed a NaCl salt concentration-dependent catalysis under our experimental conditions. For both substrates, 100 mM NaCl leads to $\sim$1.3-fold rate increase in oxime formation, while by using 1 M and 3 M of NaCl the rate enhancement further increased to $\sim$2.5-fold and $\sim$4-fold (Figure
2.8c). $^1$H NMR analysis with higher salt concentration was not performed due to the precipitation of the products.

However, the substrate concentration used in $^1$H NMR analysis is around milli-molar (mM) scale, which is significantly higher than the substrate concentration in many bioconjugation reactions. Thus we further explored the NaCl catalysis oxime formation with a substrate concentration in the micromolar (µM) range. The pseudo-first-order reaction kinetics carried out using 32 µM 4-nitrobenzaldehyde and 1 mM methoxyamine in phosphate buffer (PB, pH 7.4) containing different concentrations of NaCl (Figure 2.8d), and convention of the reaction was monitored by UV-vis spectroscopy. As shown in Figure 2.8d, reaction rate increased by ~1.7-fold in the presence of 10 mM NaCl, and the rate further increased to ~2.0-fold and ~7.3-fold in the presence of 100 mM and 1 M NaCl, respectively. It is noteworthy that a rate increase by ~20 fold was observed in the presence of the combination of 1 M NaCl/1 mM aniline. All these results clearly prove the catalytic capability of saline in oxime formation with both keto and aldehyde substrate at neutral pH.

### 2.1.2.2 Computational analysis

![Diagram](image)

**Figure 2.9** The proposed mechanism of salt-catalysed oxime formation. (a) Reaction scheme of salt-stabilized transition states. (b) Optimized geometries of the molecule showing two possible O-protonation sites (-OR and -OH) in the presence (ii and iv) and absence (i and iii) of NaCl.
We believe saline accelerated oxime formation by stabilizing the charged hemiaminal intermediate. To prove our hypothesis, we performed computational analysis of charged intermediate 22 and 23 with and without NaCl (Figure 2.9). As described previously in section 2.1.1, the formation of intermediate 22 is more favourable than intermediate 23 in the absence of catalyst due to the $\alpha$-effect of methoxy oxygen. Electronic structure calculation result reveals that 23 is slightly favourable than 22 with an energy difference of 2.4 kcal/mol (Fig. 2.18b) with the absence of NaCl. While in the presence of NaCl, the intermediate 23 is ~9-fold more stable than 22, and the energy difference increased to 20.8 kcal/mol.

We also analysed charge accumulation on the ether oxygen in intermediate 22 and hydroxyl oxygen in intermediate 23 to explore the protonation and charge transfer process. The charge in intermediate 23 is -0.77e- and -0.81e- in the absence and presence of NaCl, respectively. On the other hand, the charge in intermediate 22 is -0.47e- and -0.48e- with absence and presence of NaCl, respectively. It is clearly revealed that NaCl induces a sizeable charge difference in intermediate 23 that could attribute to salt out effect, but charge difference in intermediate 22 is negligible. In addition, intermediate 23 has lower negative charge than intermediate 22, which indicates intermediate 23 is preferable for protonation. It is noteworthy that an interaction between chloride ion of NaCl and hydroxyl hydrogen in intermediate 23 was observed, and this interaction is not observed in intermediate 22. This interaction helps the stabilization of intermediate 23, which is consistent with the experimental observation of saline catalysis in oxime formation. Salting out effect could be another reason of saline in accelerating oxime formation, which increased the local concentration of substrate.

2.1.2.3 Oxime ligation on cellular surface

We then performed salt accelerated oxime ligation on the cell surface of human colon cancer cell line (HCT116 cells). First, sialic acid residues on the cell surface were oxidized by NaIO$_4$ to introduce aldehyde moiety. Then a fluorescein-functionalized aminooxy residue was used as a nucleophile to perform the oxime reaction on the cell surface in PB (pH 7.4) with aniline, aqueous NaCl or a combination of aniline and NaCl as catalysts. The progress of the oxime reaction on the cell surface was monitored using confocal microscopy.
Figure 2.10 Oxime labelling of periodate treated HCT116 cells as analysed by fluorescence microscopy and flow cytometry using aminooxy-FITC as a nucleophile with different catalysts namely, PB as control, aniline, NaCl and NaCl having aniline as co-catalyst. (a) Confocal microscopy of HCT116 cells is indicating oxime labelling efficiency with different catalysts. All cells were fixed with 4% formaldehyde, and the nucleus was stained with DAPI after oxime reaction. (b) Flow cytometry analysis of FITC-labelled HCT116 cells with different catalysts as mentioned above. (c) Extracellular labelling and intracellular trafficking of oxime labelled cell surface receptor as analysed by AmnisFlowSight® flow cytometer.

Surprisingly, these experiments revealed that aniline did not stimulate significant oxime ligation on the cell surface in PB buffer as previously reported in PBS buffer (PB containing extra salt).65 On the other hand, a combination of aniline and salt stimulated efficient labelling on the cell surface (Figure 2.10a). This clearly proves that the salt in the buffer catalyzes oxime formation at physiological pH.

We then analyzed the bioconjugation efficiency by fluorescence-activated cell sorting (FACS) experiments (Figure 2.10b). These experiments revealed that the cell surface oxime ligation was not successful in the absence of any catalyst. However, significant labelling was observed in the group having NaCl or NaCl/aniline combination as catalysts.
Corroborating with the confocal microscopic analysis, we did not observe any cell surface labelling in the control group without any catalysts (Figure 2.10b). On the contrary, both salt and NaCl/aniline combination group showed significant labelling which displayed two distinct populations of labelled cells. The single cell image obtained from FACS analysis revealed that salt group and NaCl/aniline combination group displayed efficient cell surface labelling with some intracellular localization of the conjugated fluorophore (Figure 2.10c). While the control groups (PB and PB with aniline), displayed only intracellular trafficking of the fluorophore (Figure 2.11). Of note, the cells that did not have any cell-surface aldehyde groups (i.e., cells without periodate treatment) did not show any intracellular localization of fluorophore or extracellular labelling of cells (data not shown). These experiments clearly indicate that salt indeed influences oxime reaction kinetics on cell surfaces at physiological pH.

**Figure 2.11** Cell surface versus intracellular oxime labelling of HCT116 cells.
2.2 Tunable hydrazone cross-linking hydrogel

Hydrazone and oxime crosslinking hydrogel have been used as extracellular matrix (ECM) mimic scaffold for tissue regeneration attributed to its efficiency, selectivity and mild reaction conditions. However, excess and uncontrolled swelling of those hydrogels in vivo is a major challenge for biomedical application. In this thesis, a simple and site-selectivity aldehyde modification method was designed to introduced aldehyde moiety to hyaluronic acid, chondroitin sulphate and heparin under mild conditions, without interfering their biological functions (Paper III). After that, new hydrazone chemistries were designed and utilized to synthesize a hyaluronic acid hydrogel as BMP-2 carrier, which exhibited extraordinary hydrolytic stability, controlled degradation, good mechanical strength and controlled swelling (Paper IV). This hydrazone has also been used to develop hydrazone cross-linked ECM mimic hydrogels with the tunable release of growth factor (Paper III).

2.2.1 Synthesis of aldehyde-modified GAGs

Aldehydes are important bioorthogonal receptors for bioconjugation reactions. It can react with hydrazide and aminooxy to form a covalent bond that undergoes hydrolysis at acidic pH while stable at physiological conditions. Those linkages have been used to functionalize numerous bioactive substances for drug delivery, scaffold design, imaging and diagnosis. Although aldehyde exists in some biological molecules, for instance reducing sugars, functionalize biomolecules with aldehyde moieties remains as a prerequisite for various bioactive molecule conjugations.

Aldehyde groups can be introduced to biomolecules by the enzyme. For instance, galactose oxidase can oxidize D-galactose to aldehyde, which is important for glycoprotein modification. Aldehydes can also be introduced to cysteine containing proteins or peptides by farnesyl transferase in the presence of farnesyl pyrophosphate derivatives. These enzyme-based modification methods have good site-specify and efficiency, but they are strict to substrates with a certain structure and thus have limited applications.

Glycosaminoglycan contains various polymeric carbohydrates that are essential in living organisms, such as HA, CS, HP, cellulose and so on. They are major components of extracellular matrix and play important roles in structural support and regulating cellular activities. Commonly used methods to prepare aldehyde-modified polysaccharide include site-specific ozonolysis and reduction of enzyme degradation product, hydrolysis of an acetal protected aldehyde and radical-based oxidation methods. However, those techniques utilize harsh reaction conditions and lead to the decrease of molecule weight.
Periodate oxidation is another commonly used method to modify polysaccharide with aldehyde functional group by cleavage of the C2-C3 vicinal diols of carbohydrate. This method can be performed under the physiological condition and comparable to living cells. However, it results in the opening of disaccharide sugar ring backbone and the loss of biological functions of glycosaminoglycan. Therefore, design mild and efficient methods to introduce aldehyde moiety to polysaccharide without interference its biological function is essential for biomedical applications.

### 2.2.1.1 Synthesis of aldehyde-modified GAGs

Rate limiting steps of oxidized oxidation is the formation and disproportionation of cyclic iodate ester intermediate. (Scheme 2.4a). The reaction follows second-order reaction rate kinetics and is slow with trans-vicinal diols, while the reaction is fast with flexible diol as the substrate. Of note, C2-C3 vicinal diols in HA, HP and CS are in trans-geometry and are not favoured for the formation of the cyclic intermediate with periodate. Therefore, we introduced flexible amino glycerol that contains freely rotatable C-C bond between the hydroxyl groups to GAGs to promote site-selective and efficient aldehyde formation. This modification was achieved by amide formation between amino glycerol and the carboxylate HA, HP and CS. The reactions were performed at pH 6 following standard EDC coupling protocol in the presence of HOBr, and a large excess of amino glycerol was used to promote efficient substitution. Amino glycerol modified GAGs (Scheme 2.4b, 25a-c) were purified by dialysis.

Aldehyde modified GAGs were synthesised by oxidizing the diol-modified GAGs (Scheme 2.4b, 25a-c) with 1 equivalent of sodium periodate.
(with respect to disaccharide repeating units) for 5-10 minutes, and the excess of periodate was quenched by ethylene glycol. Desired products were purified by dialysis. The degree of aldehyde substitute was determined by \(^1\)H NMR.\(^\text{67}\)

![HA-amino glycerol and HA-CHO](image)

**Figure 2.12** \(^1\)H NMR of HA-amino glycerol and HA-CHO

### 2.2.1.2 Kinetics of periodate oxidation

![Standard curve of periodate absorbance at 290 nm](image) ![Periodate oxidation kinetic plot](image)

**Figure 2.13**

\(a\)) Standard curve of periodate absorbance at 290 nm. \(b\)) The periodate oxidation kinetic plot for amino glycerol modified HP with 20% modification.

However, analysis of \(^1\)H NMR of heparin is difficult due to the random sulphate distribution. In addition, the diol modified GAGs have similar \(^1\)H NMR spectrum with native GAGs, thus the degree of diol modification could not be obtained from NMR spectrum (Figure 2.12). In order to solve this problem, we used a new method to evaluate the synthesis and oxidation of GAGs derivative. Here, we monitored NaIO\(_4\) consumption during the oxidation by monitoring UV absorbance at 290 nm.\(^\text{69}\) A standard curve of NaIO\(_4\) absorbance at 290 nm was made first, and the linear plot was obtained for NaIO\(_4\) concentration between 0.2 mM to 7 mM (Figure 2.13a). To perform the oxidation kinetics analysis, 5 mM of sodium periodate and 5 mM of diol modified GAGs (with respect to the disaccharide repeating unit)
were reacted in distilled water in quartz cuvettes, and UV absorbance was monitored at different time points.

Table 2.2 Optimization of periodate oxidation

<table>
<thead>
<tr>
<th></th>
<th>Time (min)</th>
<th>Modification(%) -NMR</th>
<th>Modification(%) -UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-CHO</td>
<td>5</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9</td>
<td>8.5</td>
</tr>
<tr>
<td>HP-CHO</td>
<td>5</td>
<td>Not determined</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Not determined</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Not determined</td>
<td>11.1</td>
</tr>
<tr>
<td>CS-CHO</td>
<td>5</td>
<td>10</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

**Figure 2.14** Kinetics plots showing the percentage of periodate left at different time points a) HA, b) HP, c) CS.

Periodate oxidation of native GAGs is slow and consumption of NaIO₄ is negligible after 2 hours reaction (less than 3%, Figure 2.14). In contrary, oxidation of diol modified GAGs are significantly fast and finishes within several minutes. The oxidation rates for diol modified HA, HP and CS are around 5 minutes, 40 minutes and 10 minutes, respectively. As expected, the percentage of aldehyde modification obtained from UV-spectroscopy and ¹H NMR analysis are quite close (Table 2.2). The concentration of periodate remaining in the reaction solution is plotted against time (Figure 2.14). The oxidation reaction fits with second-order reaction kinetics and a representative plot fitting is shown in Figure 2.13b.

As shown in Table 2.3, the oxidation rate of amino glycerol modified GAG is nearly 4 orders of magnitude faster as compared to the native GAG, which indicates a fast and site-selective aldehyde modification. It is also observed that the oxidation kinetics of diol modified HA is faster than diol modified HP and CS. We believe the negatively charged sulphate groups on CS and HP exclude the periodate anion from the domain of the polysaccharide attributed to the significant electrostatic exclusion and steric hindrance.
Table 2.3 Second-order rate constant for periodate oxidation of native and diol modified GAGs

<table>
<thead>
<tr>
<th>Polymer</th>
<th>k_{2obs} (L•mol^{-1}•s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>1.1×10^{-4}±2.2×10^{-5}</td>
</tr>
<tr>
<td>HA-10% CHO</td>
<td>0.72±0.042</td>
</tr>
<tr>
<td>HA-20% CHO</td>
<td>0.66±0.041</td>
</tr>
<tr>
<td>HP</td>
<td>7.7×10^{-4}±4.72×10^{-5}</td>
</tr>
<tr>
<td>HP-20% CHO</td>
<td>0.21±0.042</td>
</tr>
<tr>
<td>HP-50% CHO</td>
<td>0.22±0.02</td>
</tr>
<tr>
<td>CS</td>
<td>1.2×10^{-4}±1.54×10^{-5}</td>
</tr>
<tr>
<td>CS-10% CHO</td>
<td>0.24±0.021</td>
</tr>
<tr>
<td>CS-30% CHO</td>
<td>0.23±0.05</td>
</tr>
</tbody>
</table>

2.2.2 Synthesis of hydrazide modified HA

Hydrazones and oximes possess higher hydrolytic stability as compared to imine linkage. However, hydrazones and oximes are not totally resistant to hydrolysis. The linkage cleaves gradually at physiological pH, while undergoes fast hydrolysis at acidic pH. Several methods have been designed to improve hydrolytic stability of hydrazones and oximes. The aromatic aldehyde or aromatic aldehyde with boronic acid substitution forms hydrazones that possess good hydrolytic stability\textsuperscript{18, 70}, but this approach requires specific substrate structures and thus not suitable for many applications.

\[ \text{Scheme 2.5 Hydrolysis mechanism of hydrolysis of hydrazone, oxime and imine} \]

Hydrolysis of the carbon-nitrogen double bonds starts from the protonation of imine-forming nitrogen\textsuperscript{N1}, followed by nucleophilic attack of water (Scheme 2.2). Since the neighbouring heteroatom X in hydrazones and aminoxy has higher pKa as compared to \textsuperscript{N1}, heteroatom X get protonated first and diminishes the nucleophilic attack of water. Thus, a promising strategy to improve hydrolytic stability of hydrazones and oximes is to modify the electronic density of \textsuperscript{N1} to prevent its protonation.

Here, we fine-tuned the electronic density of \textsuperscript{N2} (C=\textsuperscript{N1}-\textsuperscript{N2} - \textsuperscript{R1}) by changing functional groups \textsuperscript{R1}. Three different hydrazides namely
carbodi hydrazide (CDH), adipic dihydrazide (ADH) and oxalyl dihydrazide (ODH) modified HA were prepared following EDC coupling strategy in the presence of HOBt. 10 fold excess of dihydrazide was used to eliminate cross-linking or loop formation. The desired products were purified by dialysis and the purity was confirmed by $^1$H NMR. The extent of hydrazide substitution was determined quantitatively by UV spectroscopy using trinitrobenzene sulfonic acid (TNBS) assay. The modification was kept at a low level (7-8 %) to maintain the biological activity of HA.

Figure 2.15 $^1$H NMR of HA-CDH, HA-ADH and HA-ODH

To investigate the hydrolytic stability of CDH-hydrazone, ADH-hydrazone and ODH-hydrazone were synthesized by reacting 4-hydroxybenzaldehyde with the corresponding HA-hydrazide derivatives. Hydrolysis analysis was performed by dialysis the three hydrazones at pH 5, and the amount of released 4-hydroxybenzaldehyde was determined by UV-spectroscopy at 285 nm at different time points.

Figure 2.16 Hydrolytic stability of 4-hydroxybenzaldehyde hydrazone at pH 5.0 as determined by UV spectroscopy.
Interestingly, the three types of hydrazone exhibited significant differences in hydrolytic stability. ADH-hydrazone and ODH-hydrazone released 50% of aldehyde within 8 hours and 20 hours, respectively, and released 95% of aldehyde within 20 days (13 days for ADH-hydrazone and 17 days for ODH-hydrazone); while CDH-hydrazone exhibited much slower release and 50% of aldehyde was released at 305 hours (13 days) and 88% released at 104 days.

2.2.3 Hydrogel preparation and characterization

2.2.3.1 Hydrogel with tunable stability
To investigate the impact of hydrolytic stability on the hydrazone cross-linking on hydrogel swelling and degradation, we prepared hydrazone cross-linking HA hydrogel. HA-aldehyde (150 kDa, 8% modification) and HA-hydrazide (150 kDa, 7-8% modification) were dissolved in 10 mM PBS (pH 7.4) separately to reach a concentration of 16 mg/mL, then 250 µL of HA-aldehyde solution was mixed with the same volume of HA-hydrazide solution. Gels were incubated for 24 hours to complete crosslinking.

Scheme 2.6 Structure of the three types of hydrazide cross-linked hydrogel.
The conversion of the crosslinking reaction was determined by Fourier transform infrared (FTIR) spectroscopic analysis of lyophilized hydrogels. In all the three hydrogel’s IR spectrum, the aldehyde peak at 1729 cm\(^{-1}\) disappeared, and the peak corresponding to C=N stretching of hydrazone at 1618 cm\(^{-1}\) appears. These indicate the efficient hydrazone linkage formation (Figure 2.17a).

### Table 2.4. Rheological data of HA-hydrogels.

<table>
<thead>
<tr>
<th>HA-gel</th>
<th>modification%</th>
<th>G’ (Pa)</th>
<th>G'' (Pa)</th>
<th>tan δ</th>
<th>Mc (kg/mol)</th>
<th>ξ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH-gel</td>
<td>7</td>
<td>1196 ± 65</td>
<td>3.1 ± 0.3</td>
<td>0.0026</td>
<td>33</td>
<td>15.1</td>
</tr>
<tr>
<td>ODH-gel</td>
<td>8.2</td>
<td>956 ± 67</td>
<td>8.0 ± 0.84</td>
<td>0.0083</td>
<td>41</td>
<td>16.2</td>
</tr>
<tr>
<td>ADH-gel</td>
<td>7.4</td>
<td>297 ± 25</td>
<td>5.4 ± 0.84</td>
<td>0.018</td>
<td>129</td>
<td>23.9</td>
</tr>
</tbody>
</table>

Rheological analysis of hydrogel was performed using frequency sweep from 0.1-10 Hz with a constant normal force of 30 mN. As shown in Table 2.4 and Figure 2.17b, all the hydrogels are efficiently cross-linked. Since the loss tangent (tan δ = G’’/G’, G’’ is loss modulus and G’ is storage modulus) is below 1, which indicates the gels are more elastic materials rather than...
viscose solutions. In addition, though HA-CDH has lower modification (7 %) as compared to HA-ODH (8.2 %) and HA-ADH (7.4 %), CDH-gel exhibited higher stiffness than the other two gels. In addition, CDH-gel has smaller average mesh size ($\xi$) and average molecule weight between crosslinking (Mc) than ODH-gel and ADH-gel, which reveals a more compact crosslinking network in CDH-gel than other gels. This difference also exhibited in their hydrolytic and enzymatic stabilities.

We then evaluated the stability of the hydrogels using hydrolysis analysis. Hydrolysis of the hydrogel was carried out by immersing 500 µL of the hydrogel in 6 mL of different buffers, and weight changes of the gels were monitored at different time points. For the enzymatic degradation analysis, 500 µL of hydrogel was incubated in 3 mL of 10 mM PBS (pH 7.4) that containing 25 U hyaluronidase /mL. As shown in Table 2.4, and Figure 2.17c and 2.17d, ADH-gel and ODH-gel hydrolyzed completely within one month at neutral pH, while CDH-gel retained the morphology after 4 months under the same swelling condition. Surprisingly, CDH-gel exhibited extraordinary resistance to hydrolysis even at acidic pH, where the hydrolysis of hydrazone is usually fast. ADH-gel and ODH gel survived for 1.5 days and 2.5 days, respectively, while CDH-gel survived for 14 days at pH 5.0. In addition, CDH-gel also displayed superior stability in the presence of the enzyme.

![Figure 2.18 Major resonance structure of different HA hydrazones.](image)

The extraordinary hydrolytic stability of HA-CDH hydrazone (C=N$^1$-$N^2$-(C=O)-N$^3$H) is attributed the specific urea-type structure (Scheme 2.6a), where the positive charge of $N^2$ is delocalized. And the resonance structure
also reveals that the $\alpha$-effect of neighbouring heteroatom $N^3$ provide extensive resonance stabilization in CDH-hydrazone (Figure 2.18).

Figure 2.19 Histological examination of ectopic bone. The representative cross-sections were stained with (a) hematoxylin/eosin (H&E). (b) Masson’s trichrome. (c) picrosirius red. (d) Schematic outline of the in situ PLA strategy showing: (i) detecting RECA-1 primary antibodies; (ii) close proximity of oligonucleotide-ligated secondary antibodies allows a rolling-circle amplification (RCA); (iii) detection of the RCA product by a fluorescently labelled probe. (e) In situ PLA detection of anti-RECA-1 in histological samples. Scale bars (a-b) 100 $\mu$m, (c) 500 $\mu$m and (e) 10 $\mu$m.

HA-CDH gels are very attractive in drug delivery application attributed to the retained biological function (attributed to low modification of HA structure), mild crosslinking condition, excellent hydrolytic and enzymatic stability, and highest mechanical strength. Cell viability study (using MTS assay) with HA-derivatives and their hyaluronidase degraded fractions indicates that HA-derivatives and their degradation products are not cytotoxic. Therefore we investigated the capability of HA-CDH gel as a carrier of the therapeutic protein recombinant human bone morphology protein 2 (rhBMP-2), which is known to induce bone regeneration in vivo. The bone regeneration was performed using subcutaneous model. 200 $\mu$L of gels with or without 4 $\mu$g of rhBMP-2 were injected subcutaneously to rat’s lumbar at a minimal distance of 15 mm gap. After 7 weeks, rats were sacrificed and histological evaluation was performed. Surprisingly, we observed the formation of well-mineralized bone with abundant adipose tissue using a low dose of BMP, as compared to normally used dose around 600 $\mu$g/mL. In addition, we also observed oriented collagen matrix formation in the new bone. The blood vessel formation is also observed using a technique named proximity-ligation assay (PLA).
2.2.3.2 Hydrogel with tunable release of growth factor

Although HA-CDH hydrogel revealed extraordinary stability and drug delivery capability, lacking the protein binding site is a drawback of this material. Therefore we attempted to improve the protein binding ability of HA-CDH hydrogel by combining HA with other GAGs that have the specific protein binding ability. Here we chose sulphated disaccharide GAG, HP and CS, to develop three types of injectable hydrogels, named HA-HA gel, HA-HP gel and HA-CS gel. The hydrogels were prepared following a similar protocol as described in Section 2.2.3.1. In general, GAG-aldehyde and HA-CDH were separately dissolved in 10 mM PBS (pH 7.4) to reach a concentration of 16 mg/mL. HA-HA hydrogel was prepared by mixing HA-aldehyde and HA-hydrazide with 1:1 volume ratio and hydrogels were incubated overnight to complete crosslinking. For HA-HP gel and HA-CS gel, 50% of HA-aldehyde was replaced by the same volume of HP-aldehyde of CS-aldehyde, respectively.

The conversion of the crosslinking reaction was determined by FTIR spectroscopic analysis of lyophilized hydrogels. The disappearance of the aldehyde peak at 1729 cm\(^{-1}\) and appearance of C=N stretching of hydrazone at 1618 cm\(^{-1}\) indicates the efficient hydrazone linkage formation (Figure 2.20a).

**Figure 2.20**

- **a)** ATR-FTIR spectrum of aldehyde-modified GAGs and hydrogels.
- **b)** Rheological analysis of hydrogels before and after swelling. Frequency sweep of 0.1 – 10 Hz.
- **c)** Percentage swelling ratio of hydrogels as a function of time.
- **d)** Hyaluronidase mediated enzymatic degradation of hydrogels with time. All the experiments were performed in triplicate.

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Table 2.5 Rheology data of hydrogels.\textsuperscript{[a]} Hydrogels after 24 hours swelling

<table>
<thead>
<tr>
<th>Sample</th>
<th>G'(Pa)</th>
<th>G''(Pa)</th>
<th>Mc (kg/mol)</th>
<th>ξ (nm)</th>
<th>sw%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-HA</td>
<td>1978±48</td>
<td>3.16±0.6</td>
<td>19.98</td>
<td>12.8</td>
<td>13±2.8</td>
</tr>
<tr>
<td>HA-HP</td>
<td>1474±58</td>
<td>4.7±0.8</td>
<td>26.8</td>
<td>14.1</td>
<td>23±0.7</td>
</tr>
<tr>
<td>HA-CS</td>
<td>1085±31</td>
<td>2.9±0.5</td>
<td>36.42</td>
<td>15.6</td>
<td>43±1.6</td>
</tr>
<tr>
<td>HA-HA-s\textsuperscript{[a]}</td>
<td>1686±179</td>
<td>7.6±2.6</td>
<td>23.44</td>
<td>13.5</td>
<td></td>
</tr>
<tr>
<td>HA-HP-s\textsuperscript{[a]}</td>
<td>811±13</td>
<td>7.1±1.1</td>
<td>48.73</td>
<td>17.2</td>
<td></td>
</tr>
<tr>
<td>HA-CS-s\textsuperscript{[a]}</td>
<td>611±53</td>
<td>3.9±1.1</td>
<td>64.68</td>
<td>18.9</td>
<td></td>
</tr>
</tbody>
</table>

Swelling of hydrogels was performed by immersing hydrogels in 10 mM PBS pH 7.4 and the weight of hydrogel was recorded at different time interval. All the gels displayed fast swelling initially which reached equilibrium after 1 day. Among those hydrogels, HA-HA gel exhibited controlled swelling of ~13%, while HA-HP gel and HA-CS gel exhibited a swelling ratio of 23% and 43%, respectively. Rheology tests were performed on hydrogels before and gels after 24 hours (Figure 2.20b) swelling in 10 mM PBS (pH 7.4), and HA-CDH gel exhibited a more rigid network with the storage modulus of ~2000 Pa before swelling and ~1700 Pa after swelling. HA-HP gel and HA-CS gel are softer and exhibit less resistance to swelling, which leads to the significant loss of rigidity after 24 swelling (Figure 2.20c, Table 2.5). We believe this difference is attributed to the differences in size and structure of the GAGs. The molecular weight of HA used in research is 150 kDa, while CS and HP have a molecular weight of 50kDa and 15 kDa respectively. Meanwhile, the sulphate group in HP and CS backbone can assist the hydrolysis of hydrazone linkage as the intramolecular proton donor. Since HA-HA gel has smaller average mesh size (ξ) and average molecule weight between crosslinking (Mc), the compact network also protected the degradation by hyaluronidase (Figure 2.20d).

Figure 2.21 Release profile of proteins in different gels: a) FGF-2; b) BSA.

Finally, we investigated the capability of those hydrogels in controlled release of two therapeutic proteins, FGF-2 and BSA. FGF-2 is an important growth factor that has specific affinity to HP and Heparan sulphate, while the biocompatible protein BSA lacks the specific binding to GAGs. Hydro-
gels for the protein release study were prepared as described above. For BSA release, 250 µg of BSA was loading to 250 µL of hydrogels and then immersed in 1.5 mL of 10 mM PBS (pH 7.4) at room temperature, the release was monitored by UV-vis spectroscopy at 489 nm at different time points. For FGF-2 release, 250 µL of hydrogels containing 75 ng of FGF-2 were incubated in 1.5 mL of 10 mM PBS (pH 7.4) containing 0.5% (w/v) BSA at room temperature, and the released FGF-2 was quantitatively analysed using ELISA (enzyme-linked immunosorbent assay). Interestingly, we observed a fast release of FGF-2 in HA-HA and HA-CS hydrogel and a constant release over 14 days in HA-HP. BSA, on the other hand, did not show any substantial difference in the release profile in different hydrogel systems (released within 24h). These observations are consistent with our anticipant outcomes since HP has been reported to specific binding to FGF-2 and stabilize it in ECM.53 This simple composition tuned protein release hydrogel system would be a powerful tool as protein carrier in tissue engineering applications, where the requirement of protein release rate varies in different therapeutic methods.
Bioconjugation reactions have wide applications in the biological and biomedical field. Different reaction kinetics and product stabilities are required for diverse biomedical applications, such as reversible linkage for pro-drug synthesis, stable conjugation for tracer and imaging applications, and degradable linkages for scaffold designs. Therefore, designing a bioconjugation chemistry that allows easily adjustable reaction kinetics and product stability is highly demanded both for academic and industry. Dynamic covalent chemistry has good potential in bioconjugation application due to the reversibility and easily adaptable reaction kinetics and linkage stability. Among all the dynamic covalent chemistries, hydrazone and oxime are of particular interest. The reactions possess chemoselectivity, efficiency, atom economical, and can be performed under mild reaction conditions.

In order to optimize the bond stability in the hydrazone and oxime chemistry needed for bioconjugation applications, we investigated the mechanism and catalysis of oxime chemistry with aldehyde and ketone substrates under physiological conditions. We performed a pseudo-first order reaction kinetics in the presence of various catalysts. The inefficiency of aniline catalysis in ketone-based oxime chemistry is attributed to the α-effect of oxygen in aminooxy. We discovered that the carboxylate catalysis could overcome this α-effect by the formation H-bond with the key intermediates which drive the reaction towards the oxime formation. Carboxylates could also activate aniline catalysis in ketone-based oxime formation at neutral pH. The efficient cell surface labelling experiment verified the catalytic role of acetate as a versatile and biocompatible catalyst that exhibits similar catalytic efficiency as compared to aniline.

We also investigated the role of saline as a catalyst in oxime reaction. Kinetics analysis showed that saline accelerated oxime reaction through a concentration-dependent manner by the stabilizing of positively charged intermediate. This stabilization was further verified by the computational studies.

Finally, the dynamic covalent chemistry was utilized to design hydrogels for regenerative medicine applications. In order to perform hydrazone and oxime-based bioconjugation reactions, biomolecules (glycosaminoglycans) were functionalized with aldehyde and hydrazide groups. The aldehyde is an important bioorthogonal acceptor that has been used to conjugate biomolecules. The conventional method to generate aldehyde functional
group in GAGs were by periodate mediated oxidation of sugar rings. Such oxidation is detrimental to these polymers as they lead to fragmentation and loss of bioactivity. Therefore a mild and efficient strategy to graft aldehyde moieties without destroying the structural integrity or bioactivity of these polymers is needed. Here, we have developed a mild and site-selective method to synthesise aldehyde modify glycosaminoglycan. Kinetics study clearly proves the site-selectivity of this modification. After successful synthesis of aldehyde-modified GAGs, we attempted to design a hydrazone with good hydrolytic stability and controlled swelling. Three different types of hydrazone were synthesised and their hydrolytic stability was investigated under different conditions. Among them, carbodihydrazide based hydrazone linkage (HA-CDH) exhibited extraordinary resistance to acid catalyzed hydrolysis as compared to the other two hydrazones. HA-CDH hydrogel exhibited remarkable resistance to swelling under various conditions (pH 5, 7.4 and 9), and the compact cross-linking network also exhibited controlled enzymatic degradation. Inspired by the remarkable properties of HA-CDH hydrogel, we investigated the possibility of utilizing it as a carrier of the therapeutic protein bone morphogenetic proteins-2 (BMP-2) for bone regeneration. In vivo studies utilizing HA-CDH hydrogel as an efficient carrier of BMP-2 revealed the formation of a mature bond with well-oriented collagen (similar to neo-bone) in the presence of a significantly reduced dose of the growth factor.

In summary, mechanism and catalysis of oxime reaction under physiological pH were investigated. Carboxylate and saline have been improved to be the efficient catalyst to promote oxime formation under physiological conditions. They also promoted oxime ligation on the live cell surface. Novel hydrazone chemistry was designed and utilized to prepare extracellular mimic hydrogels with tunable hydrolytic stability, controlled swelling and enzymatic degradation for the delivery and controlled release of therapeutic proteins. These new chemistries and catalysts could extend the application of hydrazone and oxime chemistry in bioconjugation.

3.1 Ongoing studies

It is known that catalyst promotes imine formation also could be applied to accelerate cleavage of imine. Since saline accelerated oxime formation under physiological pH through the stabilization of charged intermediate, and the hydrolysis of imine also involves the charged intermediate formation, we attempt to explore the catalysis of reversible imine reaction. Saline could also be applied to catalysis hydrazone and oxime is crosslinking hydrogel formation under the physiological condition. We are planning to investigate the influence of saline tuned hydrogel cross-linking kinetics on cell behaviour.
4 Acknowledgement

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Oommen Varghese for your guidance over the past few years. Thank you for accepting me as a member of your research team and giving me the opportunity to work with you as a PhD student. You are always patient and supportive. Whenever I need help in research and writing thesis, I can always get your immediate help. Thank you for your trust and the freedom you gave in my research. Your strong sense of responsibility, perseverance and passion for make me impressed. I am lucky to have you as my supervisor. Thank you very much!

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För biokonjugering behövs dock i många fall en förbättring. Vi studerade därför i detalj mekanismen och katalysen i oximreaktionerna med aldehyd- och keton-substrat under fysiologiska betingelser. Vi utförde katalys erad reaktioner med en pseudo-första ordningens reaktionskinetik. Ineffektiven hos tidigare rapporterad anilinkatalys i ketonbaserade oximreaktionerna tillskrivs $\alpha$-effekten av syre i aminooxim. Vi upptäckte att karboxylatkatalys kunde övervinna denna $\alpha$-effekt genom formationen av väte-bindningar med reaktionsintermediärerna vilket driver reaktionen mot oximbildning. Vi fann också att karboxylater kan aktivera anilinkatalys i ketonbaseradoximbildning vid neutralt pH. Vid ett cell-ytmärkningsexperiment demonstrerade vi effekten av acetat som en mångsidig och biokompatibel katalysator med jämförbar katalytisk effekt som med toxiskt anilin.

Vi studerade också effekten salt (NaCl) som katalysator i oximreaktionen. Kinetikanalys visade att saltlösning accelererar bildning av oxim, och att kinetiken var koncentrationsberoende. Högre jonkoncentration stabiliserar den positivt laddade intermediären och sänker därmed aktiveringsenergin för reaktionen. Denna stabilisering verifierades även med beräkningsstudier.

Slutligen användes den dynamiska kovalenta kemin för att designa hydrazon- och oximbildande biokonjugeringsreaktionerna funktionaliserade biomolekyler (glykosaminoglykaner, GAG) med aldehyd- respektive hydrazidgrupper. Aldehyden är en viktig bioorthogonal acceptor som har använts flitigt för att konjugera biomolekyler. Den konventionella metoden för gene-

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