INTRODUCTION

Hydrogelators are molecules with the ability to self-assemble in water causing the solvent to immobilize. Although polymers are most commonly used to prepare hydrogels, the discovery by Gortner and Hoffman in 1921 that a small organic, amino acid-derived molecule was capable of gelling water\(^1\) prompted further exploration of this class of molecules. These investigations have revealed the advantages of low molecular weight hydrogelators (LMWG) over their polymeric counterparts, the most distinct of which is the sharp gel-to-solution (gel-sol) transition of the LMWGs.\(^2\) This property becomes important in drug and gene delivery applications. Gel-sol transitions have been achieved with a wide variety of stimuli including pH, enzymatic reaction, and light, among others.\(^2,3\) Although over 80 years have passed since their discovery, the applications of LMWGs have been developed only recently because a lack of knowledge about the mechanism of gelation prevented rational design.\(^2\) This seminar will examine recent advances in the design of low molecular weight peptide and amino acid hydrogelators, as well as the integration of stimuli responsiveness that allow the hydrogels to be used in a variety of applications.

BACKGROUND

The earliest studies on peptide hydrogelators were carried out with gelatin, a mixture of peptides obtained from the acidic or basic hydrolysis of collagen. In 1932, Olsen demonstrated that gelatin gels set at 0 °C melted faster than those set at 15 °C.\(^4\) He concluded that aggregation of the gelatin molecules is the cause of gelation.

Peptide hydrogels, like proteins, have primary, secondary, and tertiary structure.\(^3\) In polymer hydrogels (and in proteins), the primary structure consists of the polymer building-blocks covalently bonded, while LMWGs have a completely non-covalent primary structure. H-Bonding, \(\pi\)-stacking, and hydrophobic interactions are the most common modes of aggregation in LMWGs. However, H-bonding can be especially problematic, as a balance must be struck between water solubility and intermolecular aggregation. One strategy to overcome this challenge is to incorporate hydrophobic amino acids into peptide hydrogels to protect the amide groups from water so that they are free to H-bond in an intermolecular fashion.\(^5\)

The initial aggregates can further assemble into the secondary structure. The secondary structure
exists in several different morphologies including micelles, vesicles, fibers, ribbons, or sheets. The
tertiary structure involves interaction amongst the aggregates and determines whether gelation or
precipitation will occur. Long, thin fibers in the tertiary structure, as opposed to short fibers, are most
conducive to gelation.

**FMOC AMINO ACID HYDROGELATORS**

**Structure and Assembly**

Fmoc amino acids have recently attracted attention due to their identification as anti-inflammatory compounds. Since the structure of these protected amino acids also makes them good hydrogelators, they have become attractive candidates for use in biomedical applications. This class of molecules forms hydrogels through π-stacking interactions of the Fmoc groups and intermolecular H-bonding of the peptide bonds. The availability of a wide variety of amino acid side chains (aromatic, hydrophobic, hydrophilic, acidic, and basic) makes the gelation behavior of these and other amino acid hydrogelators easily modifiable.

**Enzyme-Triggered Gelation**

While thermolysin typically acts as a peptide-cleaving enzyme, under sufficient thermodynamic
pressure it can also form peptide bonds. Ulijn and co-workers discovered that gel formation on peptide
synthesis is one such thermodynamic driving force. Thermolysin is selective for the amine side of
aromatic amino acid residues (Equation 1). If the addition of thermolysin to a solution of 1 and 2
resulted in a hydrogel of tripeptide 3, the yield of tripeptide (as determined by HPLC) was higher than if
no gelation occurred. If no hydrophobic side-chain was present in the Fmoc-amino acid, the tripeptide
was not capable of gelation, and the yield of tripeptide was low.

![Chemical structure](image)

Xu and co-workers were also able to achieve enzyme-triggered gelation of water with an Fmoc
amino acid. The action of phosphatase on the sodium salt of Fmoc-tyrosine phosphate 4 revealed a
hydroxyl group which altered the balance of hydrophobicity and
hydrophilicity in the compound enough to result in gel formation (Equation 2). The driving force of hydrogelation is likely the additional H-bonding provided by the phenol -OH in Fmoc-tyrosine 5. Xu and co-workers developed this enzyme-triggered gelation into a visual assay for phosphatase inhibition in which the lack of gelation signaled enzyme inhibition.11

1,3,5-CYCLOHEXANE TRISCARBOXAMIDE HYDROGELATORS

Synthesis and Assembly

The self-assembly of 1,3,5-cyclohexane triscarboxamide 6 was studied by Hamilton and co-workers in the late 1990s.12 The crystal structure of 6 showed intermolecular stacking via H-bonding through the amides as well π-stacking of the pyridine rings.

The self-assembly of cyclohexane triscarboxamide 6 made this type of molecule a promising starting point in the search for new hydrogelators. Feringa and co-workers synthesized a wide variety of potential gelators containing the cyclohexane triscarboxamide core as shown in chart 1.5 Only those derivatives containing hydrophobic amino acids formed hydrogels, most likely due to the ability of the hydrophobic side chain to protect the amides from competitive H-bonding with water.

The crystal structure of tyrosine derivative 7 revealed that the molecules stack via intermolecular H-bonds involving the amide groups and showed that the phenyl ring folded inward to prevent the amides from H-bonding with water. The lack of hydrophobic side chains in serine derivative 8 and glycine derivative 9 causes them to be water soluble, lending support to the idea that a hydrophobic group near the amides prevents them from H-bonding with water. Phenylalanine derivatives 10 and 11 demonstrate the importance of intermolecular H-bonding. The extra H-bond donor group of 10 is thought to be responsible for its elevated gel-sol transition temperature (Tgs), some 20 °C higher than that of the analogous ester 11.

pH Responsiveness

Compounds 12 and 13 provided insight into the effect of pH on gelation. These acidic gelators
underwent the gel-sol transition upon basification and the reverse transition upon acidification. The pH at which the gel-sol transition occurred (pH$_{gs}$) was significantly higher for 12 (4.3-5.8) than for 13 (3.2-4.0) despite the fact that the carboxylic acid groups of each gelator should have the same pK$_a$. Raising the pH causes negative charges to accumulate in adjacent layers (Figure 1). If the attractive forces between these layers are strong enough, the repulsive forces can be tolerated. Since 12 has an additional peptide bond, the amount of H-bonding between the layers increases, and therefore 12 can remain a gel at higher pHs than compound 13. This demonstrates how tuning the pH-responsiveness of a gel can be achieved without changing the pK$_a$s of the pH-sensitive groups.

Two-Stage Drug Release

The potential of enzymatic degradation of pharmaceuticals is a concern when designing new drugs. The controlled release of a drug in a specific cell type by use of tissue-specific enzymes could offer a solution to this problem. Feringa and co-workers used cyclohexane triscarboxamide gelator 14 to protect a model drug from enzymatic cleavage until a stimulus (heat) was applied to dissolve the gel and release the drug.13

Hydrogelator 14 contains ethylene glycol groups on two of the amides to act as a gelating scaffold. On the third amide is an enzymatically cleavable phenylalanine functionalized with 6-aminoquinoline (6-AQ), a fluorescent model drug. The rate of peptide cleavage by $\alpha$-chymotrypsin was measured by the rate of appearance of 6-AQ by fluorescence spectroscopy. The initial rate of hydrolysis ($V_0$) of 14, measured over a range of concentrations, leveled off at the critical gelation concentration (CGC), indicating that aggregation into gel fibers effectively protects 14 from enzymatic degradation. In addition, the maximum rate of hydrolysis ($V_{\text{max}}$) of gelating 14 was significantly slower (4.1 $\mu$mol/min) than that of non-gelating carboxylic acid 15 (22.3 $\mu$mol/min).

The effect of temperature on the rates of hydrolysis of compounds 14 and 15 was also investigated. Over the temperature range of 25-45 °C, the rate of hydrolysis of compound 14 increased 1200%. The authors propose that the rate of hydrolysis increases at these high temperatures because 14
is undergoing the gel-sol transition which releases the peptide bond from the protection of the gel fibers and allows it to be cleaved by the enzyme. The same increase in temperature only caused the rate of hydrolysis of compound 15 to increase by 100%, likely due to temperature-induced rate enhancement of the reaction.

**N-ACYLGLACTOSAMINE HYDROGELS**

**Synthesis and Discovery**

While developing a solid-phase synthesis of artificial glycolipids, Hamachi and co-workers discovered that several of the glycolipid derivatives had the ability to gel organic solvents. Inspired by this unexpected result, they used solid phase synthesis for the rapid identification of hydrogels based on N-acylgalactosamine. Although several different glutamate esters were synthesized, only cycloalkylmethyl glutamate esters 16 and 17 were found to form hydrogels.

**Self-Assembly of Methylcyclohexylglutamate Derivative**

The powder X-ray diffraction of glutamate ester 16 gave two main peaks: 38 Å, which is roughly twice the molecular length of 16 and 4 Å, about the molecular thickness of the cyclohexyl ring. These data indicate that 16 assembles into a bilayer structure and that the stacking of the cyclohexyl rings in the gel is tight. The crystal structure of 16 confirmed the diffraction data. The crystal structure also revealed intermolecular H-bonding among the amides and among the sugars and water.

**Stimuli Responsive Volume Transition**

A hydrogel prepared with 16 was found to shrink on heating and to re-swell on cooling. Until Hamachi’s report in 2002, “smart” LMWGs were only known to undergo stimuli- responsive gel-sol transitions, although a similar volume transition has been reported for poly-N-isopropyl-acrylamide. Gel dissolution was attributed to the breaking of the intermolecular interactions by the given stimulus. Gel shrinkage, however, is caused by the expulsion of water from the gel matrix. The authors concluded that when heated to temperatures as high as 72 °C, the hydrogel of 16 shrinks rather than dissolves due to the extremely strong H-bonding present in the gel.

In another study, a pH responsive volume transition was observed in the two-component gel of 16 and 18 (1:10 mixture). Carboxylic acid 18 was incorporated to confer pH responsiveness on the gel. At pH 4.0, the two-component gel underwent the previously observed
volume transition at 72 °C. At pH 7.0, however, heating to 72 °C resulted in a gel-sol transition. These results were also explained by the strength of the H-bonding in the gel. At pH 7.0, the negatively charged carboxylates disrupt the H-bonding in the gel. Heating the gel to 72 °C is sufficient to break the weakened intermolecular forces and dissolve the gel. On the other hand, at pH 4.0, the fully protonated carboxylic acids retain the strength of the hydrogen bonds and the volume transition was observed at 72 °C. Changing the composition of the gel from 1:10 to 1:1 16/18 gave rise to an exclusively pH responsive volume change (i.e. no heat was required to cause gel shrinkage). 20

**Phosphate Sensing**

The development of new chemosensors for the detection of biologically relevant phosphate derivatives is a rapidly expanding field.21a-d The utilization of these chemosensors for high throughput analysis has been explored as well. Immobilization of the receptor is required for this application, but the common method of covalently immobilizing the receptor on a polymer bead or a glass surface has several disadvantages, the most detrimental of which is loss in receptor activity. Hamachi and co-workers proposed that encapsulation of the receptor in a hydrogel would be ideal for this application, as it would immobilize the receptor in a non-covalent fashion allowing it to retain its activity.22 In addition, the “semi-wet” nature of the hydrogel (water is immobilized amongst the hydrophobic fibers of the gel) provides an ideal environment for water-soluble phosphates.

To sense different biologically relevant phosphate derivatives, Hamachi incorporated fluorescent receptor 19 into the hydrogel of 16. Receptor 19 was designed to respond (in terms of wavelength and intensity of fluorescence signal) to changes in the hydrophobicity of its environment. When a hydrophilic phosphate (e.g. ATP) was added to the hydrogel containing 19, a red shift in emission maximum and a decrease in fluorescence intensity were observed. This was attributed to the phosphate-receptor pair moving to the aqueous domain of the hydrogel. When a hydrophobic phosphate was added (e.g. triphenyl phosphate), the opposite change in both emission maximum and fluorescence intensity was observed. This change was attributed to the phosphate-receptor pair moving to the hydrophobic fibers of the gel. When this assay was performed in media that did not contain both hydrophobic and hydrophilic domains (water, agarose gel), no fluorescence changes were observed.

Phosphate receptor 20 and styryl dye 21, a FRET pair having fluorescence emission peaks at 485 and 569 nm, respectively, were embedded into the hydrogel of 16.22 When a hydrophobic phosphate
(e.g. triphenyl phosphate) was added to this gel, an increase in the intensity of the 569 nm peak was observed with concomitant decrease in the intensity of the 485 nm peak. The opposite change was observed when a hydrophilic phosphate (e.g. ATP) was added.

The dye, 21, was expected to localize in the hydrophobic pockets of the gel. In the presence of a hydrophobic phosphate, the receptor/phosphate pair (FRET donor) migrates to the hydrophobic portion of the gel, in close proximity to the dye (FRET acceptor), leading to FRET enhancement. When a hydrophilic phosphate was added, the FRET donor and FRET acceptor pair move further away from each other, leading to FRET cancellation.

**Protein Microarray**

Using a concept similar to that for phosphate sensing, Hamachi and co-workers developed an assay for the activity of three N-terminus selective proteases (α-chymotrypsin, lysyl endopeptidase, and V8 protease). For this assay, compound 22, a conjugate of a hydrophilic peptide and the environmentally sensitive fluorescent probe, DANSen, was incorporated into a gel prepared with 16. The gel was prepared as a spot on a glass plate. The protease was then injected into the gel. Three different peptide-fluorophore conjugates, each containing a different amino acid at the N-terminus of the peptide were tested in this assay. When the protease added to the gel was selective for the amino acid at the N-terminus of the peptide, an increase in fluorescence emission intensity and a red shift in the emission maximum were observed. For example, in the case where the peptide had an N-terminal glutamate, only the spots where V8 protease was added changed color. The authors interpreted these results to mean that the fluorophore is moved from the hydrophilic space in the hydrogel to the hydrophobic space on peptide cleavage (Figure 2). If this is the case, then the peptide cleavage should...
result in a re-distribution of the hydrophilic peptide and hydrophobic fluorophore. The peptide should remain in the aqueous space of the hydrogel and DANsen should move to the hydrophobic fibers of the gel. This change in the environment of the fluorescent probe causes the observed change in its fluorescence spectrum. Only a slight shift in the emission maximum was observed when the cleavage reaction took place in aqueous solution.

CONCLUSION

The potential of LMWGs is just beginning to be tapped. Initial efforts to use gels of low molecular weight peptides and amino acids in drug delivery and sensing applications have not yet been pursued past the proof-of-concept stage. However the current ability to design these gelators rationally should both improve existing applications and lead to the development of new uses.

REFERENCES