Supramolecular gel-phase materials with advanced functions

Irwansyah

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NANYANG
TECHNOLOGICAL
UNIVERSITY

SUPRAMOLECULAR GEL-PHASE
MATERIALS WITH ADVANCED
FUNCTIONS

IRWANSYAH

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2014

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A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of Doctor of
Philosophy

2014

Abstract

Fabrication of new supramolecular gel-phase materials based on self-assembly of low molecular weight gelator is motivated by potential applications of supramolecular gels in diverse fields. These types of materials have found application in field of biomaterial, electronic, sensing, and template for inorganic synthesis. However, most of low molecular gelator reported required complex design and multi steps covalent practices. Hence, simple design and synthesis but at the same time possess advanced functions are needed for fabrication of supramolecular gel.

In this thesis, firstly we reported a low molecular weight organogelators (LMWOGs) based on Fmoc-amino acid for multi purposed application. The gelator, Fmoc-lys(boc)-C16 was synthesized by one step coupling between amino acid bearing chromophore, Fmoc-lys(boc)-OH, and hexadecylamine. The gelator forms a stable transparent gel in cyclohexane and hexane with ultra-low minimum gelation concentration (MGC) due to the cooperative formation of hydrogen bond and of π - π stacking of Fmoc moiety as indicated by Fourier transform infrared spectroscopy (FTIR), fluorescence and circular dichroism (CD) study. Field emission scanning electron microscopy (FESEM) image revealed that gels consist of 1D self-assembly fibrous network. We demonstrated that Fmoc-lys(boc)-C16 selectively gels organic solvent from their mixture in water. We also reported an efficient energy transfer between fluorenyl and dansyl moieties within self-assembled organogel. Energy transfer study indicates that the addition of acceptor group (octyl dansyl) decreases the emission of Fmoc group within the gel phase. Concentration dependent study

revealed that high efficiency energy transfer (~89%) between Fmoc and Dansyl was achieved in gel phase. Moreover our study also shown that inclusion of 3% acceptor causes 50% quenching of donor that make Fmoc-Dansyl system as an ideal light harvesting system. As a multi purpose organogel, we also demonstrated that Fmoc-lys(boc)-C16 gel was able to remove organic dye from solution with high efficiency (~94%).

In the second works, the formation of supramolecular hydrogel via ultra simple and facile approach using Fmoc-amino acid as building block was described. Fmoc-phenylalanine (F) was the smallest Fmoc-amino acid as building block for self-assembly into hydrogel. Our systematic studies revealed that molecular arrangement of F in self-assembly structure was driven by π - π stacking of Fmoc group and H-bond. In addition, the intermolecular interaction was stabilized by the presence of phenyl group stacking. Furthermore, to fabricate straight forward strategy for antimicrobial supramolecular hydrogel, we described a facile route for antimicrobial hydrogel without involving expensive and time consuming covalent practices. The incorporation of Fmoc-leucine (L) into F hydrogel results in antimicrobial supramolecular hydrogel that able to kill bacteria pathogen *S. aureus* with fast killing rate.

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Chapter 1 LITERATURE REVIEW

This chapter first describes the fundamental background of molecular self-assembly and supramolecular gel-phase materials. Next, the design and classification of low molecular weight gelators (LMWGs) are outlined, followed by example of the diverse applications of supramolecular gels from biomaterials to nanotechnology. In addition, peptide-based low molecular weight gelators as emerging building block for fabrication of supramolecular gels are described. Finally, research motivations and objectives as well as the organization of the thesis are presented.

1.1 Molecular Self-Assembly and Supramolecular Gels

1.1.1 Overview

Molecular self-assembly, ubiquitous process in biological system is a powerful approach for fabricating supramolecular architecture.^{1,2} Facilitated by numerous weak non covalent interactions namely hydrogen bonds, ionic bonds, and hydrophobic interaction, molecular self-assembly forms well-defined and stable hierarchical structure. The example of hierarchical structure in biological system as results of molecular self-assembly can be found in diverse systems, such as double-stranded DNA, triple-stranded helices collagen as well as complex structure of tobacco mosaic virus.³ Since supramolecular nanostructures possess key role and special function in biological system, it

then inspired researchers to fabricate new technologies which rely on self-assembly of synthetic molecule to mimic technology provide by nature. However, artificial self-assembly using synthetic molecule exhibit some challenges such as design building block that able to provide non-covalent molecular recognition to direct the molecule aggregation into well-defined self-assembly structure and at same time posses high stability at macroscopic scale.⁴

One particular form of molecular self-assembly is supramolecular gel-phased materials which are generated from entrapment of liquid (solvent) into bulk amount of entangled nanofibers network to create soft solid-like mass. In supramolecular gel-phased materials, the entangled nanofibers are resulted from one dimensional self-assembly of small molecules driven by non covalent interaction. Although gels derived from low molecular weight molecule known for long time, the supramolecular nature of these materials was poorly understood until the late of 20th century.⁵ Nevertheless, the newly “discovered” area of supramolecular gels has witnessed an enormous surge of activities in the past decade, owing to growing interest in constructing nanomaterials that have potential application in field of catalyst, optoelectronics, biomedicines, tissue engineering and light harvesting.⁶⁻⁹

1.1.2 Introduction to Supramolecular Gel-phase Materials

Gels are viscoelastic solid-like materials materials that can be found in everyday application ranging from toothpaste, hair gel, contact lenses as well as gelatin dessert.^{5,10} Gels are always consist two components: gelator as minor component and solvent as major component. The viscoelastic properties of gels

are resulted from immobilization of solvent in three-dimensional (3D) cross-linked network formed by gelator molecules.^{5,11} One substance is classified as a gel based on a visual observation of its flow characteristics or crude rheology properties.¹⁰ Flory proposed in 1974 four categories of gels based on its continuous structural: ordered lamellar structures (including gel mesophases), disorder network by covalent polymer, aggregation induced by physical interaction of polymer chain, and disordered structure by particulate.¹² Gels also can be classified according to the medium, source, constitution, and type of cross-linking that creates their 3D network which is shown in Figure 1.1.⁵ The term of supramolecular gels can be used to encompass any gelating species that uses non-covalent interaction to form gel and has been commonly used to describe gels that are resulted from self-assembly of low molecular weight compound (low molecular weight gelators, LMWGs).¹³ Supramolecular gels are usually prepared by dissolved small amount of gelator (usually 0.1-10 wt %) in an appropriate solvent followed by heating which results an uniform and supersaturated condition, and cooling to room temperature (below temperature of gelation, T_{gel}) without disturbance.^{5,14} Since supramolecular gel resulted from weak non covalent interaction, the gels can be broken by application of heat and reformed upon cooling the solution, which become advantage over polymeric gels.

Figure 1.1 Classification of Gel-Phased Materials. Reprinted with permission from reference 5. Copyright 2005 The Royal Society of Chemistry.

1.2 Design of Low Molecular Weight Gelators (LMWGs)

1.2.1 Classes of LMWGs

We have known that gel can be divided into hydrogel and organogel according to the nature of the liquid-like phase, and based on the type of components been used, gels derived from self assembly of small molecular gelator are classified into two category: single component and multi component gel. Single component gel required only one compound to gelate the solvent, whereas in multi component gel, two (or) more compound was added to solvent in order to produce supramolecular gel.¹³ To get better understanding of multi component gel, most of research group focuses on two component gel,

which can be grouped into three classes: both components necessary for gelation, both components are gelator, and one component is gelator whereas the other is additive. Those classes of two component gel are depicted in Figure 1.2.

Figure 1.2. Cartoon representation of three types of two component supramolecular gels. Reprinted with permission from reference 13. Copyright 2012 The Royal Society of Chemistry.

From the perspective of structural motifs, low molecular weight gelator can be grouped into several classes: (1) fatty acid derivatives, (2) steroid derivatives, (3) anthryl derivatives, (4) peptide based gelator, and (5) organometallic compound.¹⁵⁻¹⁹ These structural motifs are depicted in Figure 1.3.

Figure 1.3. Structural motifs of Low Molecular Weight Gelators (MLWGs). Reprinted with permission from reference 15. Copyright 1997 American Chemical Society

1.2.2 Peptide-based LMWGs

Protein and peptides serve as the major molecular scaffold material of biological world at nano-scale, micro-scale, and macro-scale.²⁰ There are 20 natural amino acids that are utilized in the synthesis of peptide and proteins in biological cell. Those amino acids which serve as building block for designed peptide provide distinct and specific interaction toward the environment due to the nature of amino acid side chain. Amino acid with basic or acid side chain allows for electrostatic interaction as well as pH responsive materials. On the other hand, amino acid with polar non charge group (hydroxyl) allows hydrogen bond and temperature responsive materials.²¹ Beside that, disulfide bridge forming amino acid (cysteine) can also be used to fabricate electrochemical responsive material based on amino acid. Figure 1.4 summaries the interaction provides by each amino acid. According to Figure 1.4, we can clearly see that the interactions between amino acids were effected by the nature of amino acid side chain. These interactions contributed to supramolecular arrangement of amino acid based molecule (peptide). Supramolecular structure based on peptide molecules can be formed when a large number of single peptide interacts with another peptide molecules via non covalent forces such as ionic, hydrogen bonding, hydrophobic as well as π - π stacking.²²

Figure 1.4. Amino acids and specific interaction they provided. Reprinted with permission from reference 21. Copyright 2006 The Royal Society of Chemistry.

Generally, self-assembly of peptide based materials fall into two categories: natural and non-natural self-assembly. Natural self-assembly of peptide use the common peptide conformation such as β -sheets (parallel and anti parallel), α -helices as well as coiled coil, whereas non-natural self-assembly covalently links amino acid to other molecules either alkyl to form an

amphiphilic peptide or aromatic group to aromatic-conjugated peptide which able to provide π - π interaction. Figure 1.5 shown amino acid structure and potential application based on self-assembly architecture.

Figure 1.5. Structure of 20 natural amino acids with their common names and possible supramolecular architectures. These supramolecular architectures find applications in biological and non-biological field. Reprinted with permission from reference 22. Copyright 2008 the Royal Society of Chemistry.

Zhang's group at MIT designed self-assembly peptide into hydrogels using ionic self-complementary peptide motif or "peptide lego".²³ They were serendipitously discovered peptide lego motif from protein in yeast.²⁴⁻²⁶ Those lego motifs have 16 amino acid residues as repeating unit motif, NH₂-AEAEAKAKAEAEAKAK-COOH (EAK16-II).²⁷ The presence of lysine (K) and glutamic acid (E) residues on EAK16-II provide ionic properties (hydrophilic) whereas alanine (A) residue provides hydrophobic properties. Zhang's group at MIT has classified the ionic self-complementary peptide into different types based on the hydrophilic pattern (charge) of amino acid residues. Peptide with type I has -++- pattern whereas peptide with type II has ---+ pattern and so on. Figure 1.6 depicted the structure of ionic self-complementary peptide motifs. These peptides undergo self-assembly to form hydrogel with low peptide concentration (0.1 to 0.5% weight of peptide) through the formation of β -sheet structure. Furthermore the same group also demonstrated that hydrogel formed from self-assembly of peptide lego motifs is able to reforming via reassembly process after being broken by sonication.²⁸ Reassembly process of peptide lego can be easily recognized by the growth of nanofiber length during the time as shown in Figure 1.7. Interestingly upon the reforming process, the mechanical properties of hydrogels increase as the length of nanofiber increase.

Figure 1.6. a) Self-complementary ionic peptide has two distinctive sides, hydrophobic side and hydrophilic side, by varying the charge of hydrophilic side there are three possible designs that can be designed. b) Examples of self-complementary ionic peptide. Reprinted with permission from reference 27. Copyright 2009 Elsevier Ltd

Figure 1.7. Time-dependent AFM images of nanofiber formed from reassembly of peptide lego RADA-1 after sonication. a) 1 min; b) 2 min; c) 4 min; d) 8 min; e) 16 min; f) 32 min. Reprinted with permission from reference 28. Copyright 2005 Proceedings of the National Academy of Sciences of the United States of America.

Recently, Hauser, et.al reported shorter peptide self-assembly than ionic self-complementary motif developed by Zhang's group.²⁹⁻³² Their work were

based on natural peptide consist only 3 to 6 amino acid residues that self-assembled in water into nanofiber to form hydrogel. Nanofiber formation was resulted from anti parallel β -sheet interaction that subsequently changes into α -helix as intermediate structure (Figure 1.8). Generally, ultra-small peptide developed by Hauser et.al consists of aliphatic non polar amino acids as tail (N-terminus) and hydrophilic amino acid (acidic, basic or polar group) as head group (C-terminus). According to their works, peptide with 6 amino acid residue has better chance to form hydrogels than shorter peptide (5, 4 or 3 amino acids residues). In addition, peptide with acidic head group (Aspartic acid and glutamic acid) formed stronger gel than peptide with neutral (serine and threonine) or basic amino acid (lysine).

Figure 1.8. Proposed mechanism of self-assembly of ultra-small peptide into supramolecular structure. Self-assembly was started with the formation of anti parallel β -sheet of two peptide chains and subsequently change into helical pairs. Reprinted with permission from reference 29. Copyright 2011 Proceedings of the National Academy of Sciences of the United States of America.

So far, we have described two groups of peptide self-assembly: peptide lego and ultrasmall peptide which are categorized as natural self-assembly motifs. Another category of peptide self-assembly is non-natural self-assembly motif which can be produced by covalently joined non peptide motif into the structure. The first non natural motif for fabrication of supramolecular gels based on peptide is peptide amphiphiles (PAs) which is pioneered by Stupp's Group at Northwestern University.³³⁻⁴⁰ In general, peptide amphiphiles (PAs) comprised two segments, peptide and non peptide segment. Non peptide segment provides hydrophobic properties which are normally resulted from long alkyl chain fatty acid. Meanwhile, peptide segment provides various structural motifs such as β -sheet segment, disulfide segment as well as charge on head group (Figure 1.9). Their work revealed that PA molecules self-assembled into ultra long cylindrical nanofibers and at certain concentration form three-dimensional network that appeared as hydrogel. According to their investigation, the internal structure of peptide amphiphile nanofiber is constructed by β -sheet structure oriented parallel to the long axis of nanofibers that pack radially from the nanofiber core.⁴¹ Furthermore, they also reported that the mechanical properties of self-assembled fibrillar networks composed of peptide amphiphile can be tuned by gelation methods.⁴² Hydrogel formed from CaCl_2 -induced self-assembly possesses higher stiffness than hydrogel formed from HCl -induced self-assembly. In addition, while study the effect of alanine and valine residue on self-assembly peptide amphiphile, the same group also reported that the stiffness of gel could be increased by introducing valine into the structure, whereas increasing the number of alanine residues decrease the

stiffness.⁴³ Furthermore, instead of using cations for neutralizing negatively charge peptide amphiphile, they also developed mixed self-assembly system involving two oppositely charge and polarity of peptide amphiphiles.^{44,45}

Figure 1.9. a) Example of peptide amphiphile structure which is consisted of peptide (segment 2, 3, 4 and 5) and non peptide segment (segment 1); b) cartoon representation of peptide amphiphile molecule; c) Peptide amphiphile self-assembled long cylinder nanofiber. Reprinted with permission from

reference 18. Copyright 2001 Science, by American Association for the Advancement of Science.

Beside those motifs for fabrication of supramolecular gels, peptide backbone also can be modified using aromatic group. N-(fluorenyl-9-methoxycarbonyl) (Fmoc) protecting group is the most commonly used aromatic for fabrication of peptide-based supramolecular gel. A number of Fmoc conjugated-peptide has been developed ranging from Fmoc di-, tri-, tetra-, and penta- peptide. Xu and co-workers reported the example of responsive supramolecular hydrogels that exhibit sol-gel transition respond toward specific biomolecules.⁴⁶ Their work is based on D enantiomer of Fmoc-dialanine hydrogels that turn into solution phase after the addition of vancomycin as ligand. Their ability to control the properties of supramolecular hydrogel using biological recognition offers new design for drug delivery system. The presence of vancomycin disturbed the delicate balance between hydrophobic interaction and hydrogen on Fmoc-D-Ala-D-Ala hydrogel and induced hydrogels disassembly, whereas L enantiomer of Fmoc-dialanine hydrogels showed no response to vancomycin (Figure 1.10). Furthermore, their supramolecular hydrogel also exhibited pH and thermal responsive behavior.

Figure 1.10. Sol-gel transformation on D enantimer of Fmoc-dialanine supramolecular hydrogel in the presence of Vancomycin. Reprinted with permission from reference 46. Copyright 2003 American Chemical Society.

Beside Fmoc-dialanine, other Fmoc-dipeptide molecule which has been studied well for supramolecular hydrogel was Fmoc-diphenylalanine (Fmoc-FF). Ulijn and co-workers proposed model for supramolecular hydrogels obtained by self-assembly of Fmoc-FF. Their model depicted the presence of anti parallel β -sheets and anti-parallel π - π stacking fluorenyl groups (Figure 1.11).⁴⁷ They also proposed self-assembly mechanism of Fmoc-FF at different pH above the critical gelation concentration with different degree of ionization as shown in Figure 1.12.⁴⁸ As we can see from Figure 1.12 translucent hydrogel formed when pH of solution decrease from 10.2 to 9.5. Further decreasing of pH value will result in aggregation and precipitation. In addition, the same group also investigated the effect of phenylalanine replacement by glycine on Fmoc-FF self-assembly and gelation behaviour.⁴⁹ Their investigation revealed

that replacing of two phenylalanine residues (Fmoc-GG) and one phenylalanine (Fmoc-FG) still form hydrogel whereas Fmoc-GF failed to form hydrogel.

Figure 1.11. Model structure for Fmoc-FF with an anti parallel β -sheet and Fmoc-stacking. Reprinted with permission from reference 47. Copyright 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Figure 1.12. Schematic representation of self-assembly mechanism of Fmoc-diphenylalanine at different pH as function of the peptide degree of ionization. Reprinted with permission from reference 48. Copyright Copyright 2009 American Chemical Society.

Another strategy for fabrication of supramolecular hydrogel using Fmoc-dipeptide is by using enzyme triggered self-assembly.^{50,51} Ulijn's group demonstrated two complementary enzymatic methods to prepare peptide-based nanomaterials: 1) subtilisin-assisted self-assembly via hydrolysis of Fmoc-dipeptide methyl esters and 2) thermolysin driven self-assembly via reverse hydrolysis as shown in Figure 1.13.

Figure 1.13. Enzymatic-assisted hydrolysis of Fmoc-peptide methyl ester converts the solution into gel phase. Reprinted with permission from reference 51. Copyright 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

The effect of amino acid residue has also been studied by using Fmoc-tripeptide. Hamley and co-workers investigated the interchange of lysine and valine residues using two types Fmoc-tripeptide: Fmoc-Val-Leu-Lys(Boc) and Fmoc-Lys(Boc)-Val-Leu.⁵² Their investigation revealed that Fmoc-Val-Leu-Lys(Boc) formed highly anisotropic fibrils whereas Fmoc-Lys(Boc)-Val-Leu formed branched fibrous structures and has lower minimum gelation concentration. These behaviors are corresponded to conformational differences, Fmoc-Val-Leu-Lys(Boc) form β -sheet structure, whereas Fmoc-Lys(Boc)-Val-Leu form unoriented aggregation and multiple sheets stacked each other. In

addition, their work also suggested that changing the sequence of amino acid residue affected the mechanical properties of resulted hydrogels as Fmoc-Val-Leu-Lys(Boc) lower modulus than Fmoc-Lys(Boc)-Val-Leu.

Supramolecular hydrogels from Fmoc-pentapeptide have been reported by Xu, et. al. with some of sequences has specific binding toward antigens and other biomolecules (Figure 1.14).⁵³ Their work revealed that the Fmoc-Fmoc interaction and hydrogen bond formation is critical for Fmoc-modified peptide self-assembly into hydrogel. According to their report, the emission spectra of Fmoc-modified peptide hydrogels appear at higher wavelength than solution which indicate the presence of eximers of fluorenyl rings.

Figure 1.14. Structure of Fmoc-Pentapeptide (R=Fmoc) which contain bioactive sequences. All of Fmoc-pentapeptide behave as gelator except

compound 3. Reprinted with permission from reference 53. Copyright 2010 American Chemical Society

1.3 Application of Peptide-based Supramolecular Nanostructured-Gel

Supramolecular gel has resulted enormous activity in constructing nanomaterials for special application, ranging from biomaterials to electronic devices. These high-tech applications of molecular gel were resulted by programmed self-assembly. In programmed self-assembly, the gelator is designed so as to contain both of self-assembling unit and functional motif that expressed at macroscopic level (Figure 1.15).⁶

Figure 1.15. Schematic illustration of design functional material through self-assembly gel-phase materials. Reprinted with permission from reference 6. Copyright 2009 the Royal Society of Chemistry.

1.3.1 Biomedical Application

The applications of peptide-based supramolecular gel on biomedical field appear as platform for regenerative medicine as well as therapeutic delivery system. Here we provide the selected examples.

Stupp and co-workers has utilized their peptide amphiphile molecule as supramolecular hydrogel for articular cartilage regeneration.⁵⁴ Their design was based on co-assembly of peptide amphiphile without binding epitopes (filler) and peptide amphiphile with binding epitope (peptide amphiphile with HSNGLPL sequence, a TGF- β 1 binding domain) as depicted in Figure 1.16. Their study indicated that nanofiber formed by those two peptides possessed high density epitopes. Kinetic release of epitopes revealed that the epitopes release from co-assemble hydrogel (peptide amphiphile and peptide amphiphile with binding epitope) was slower than hydrogel from peptide amphiphile loaded with TGF- β 1 via simple mixing. Furthermore, their in vitro and in vivo experiments indicated that co-assemble hydrogel nanofiber promote regeneration of articular cartilage. The same co-assembly method has also been demonstrated for fabrication of cytotoxic supramolecular membrane for cancer therapy.⁵⁵ The peptide amphiphile with amphipathic α -helical sequence KLAKLAKKLAKLAK was used as bioactive agent since it induced cell death and exhibited cell specificity toward breast cancer cell. Previously the same group has also developed controlled-release drug from peptide amphiphile hydrogels.⁵⁶ By placed drug model (Prodan) at different position along the backbone of peptide amphiphile (Figure 1.17) they were able to control kinetic release of Prodan. Their work revealed that controlled-release of Prodan from

self-assembly nanofiber has zero-order kinetics. However, half-life of Prodan released was affected by the position of drug in peptide amphiphile segments. Furthermore, it has been reported that the release of Prodan from peptide nanofiber by diffusion mechanism (unbound Prodan) was two times faster than Prodan covalently-joined with peptide amphiphile.

Figure 1.16. a) Chemical structure of peptide amphiphile with binding epitope; b) Chemical structure of nonbioactive peptide amphiphile which act as filler (peptide amphiphile without epitope); c) Illustration of co-assembly peptide amphiphile with binding epitope and filler into nanofiber; d) Time dependent ELISA results showing TGF-binding PA release from 6 to 72 hours. Reprinted with permission from reference 54. Copyright 2010 Proceedings of the National Academy of Sciences of the United States of America.

Figure 1.17. a) Proposed structural models of peptide amphiphile with different position of prodan (Alkyl tail shown in gray, β -sheet region in blue, charged residues in red whereas lysine-hydrazone-prodan group in green; b) Release of prodan from peptide amphiphile nanofibers. Reprinted with permission from reference 56. Copyright 2012 The Royal Society of Chemistry.

One particular interesting properties of supramolecular hydrogels based on peptide amphiphile was their ability to mimic extracellular matrix (ECM). Since ECM possesses rich arginine-glycine-aspartic acid (RGD) sequence, Stupp and co-workers reported the modification of peptide amphiphile structure by attaching RGD sequence as bioactive epitopes for cell adhesion.⁴⁰ Inspired by

degradation susceptibility of ECM by enzyme secreted by attached cell, they also designed peptide amphiphile bearing cell adhesion motifs (RGD) but at the same time bearing enzyme cleavage motif (GTAGLIGQ sequence) on peptide amphiphile structure.⁵⁷ These design enabled us to create cell responsive extra cellular matrix.

Zhang and co-worker also reported the utilization of peptide hydrogel for controlled release of functional protein. Their work is based on encapsulation variety of proteins within the Ac-(RADA)₄-CONH₂ peptide hydrogel.⁵⁸ The proteins including lysozyme, trypsin inhibitor, BSA and IgG with differing physicochemical properties and morphologies. The release kinetics suggested that proteins diffusion through nanofiber hydrogels depended primarily on the size of the protein and hydrogel nanofiber density (Figure 1.18). Moreover, secondary and tertiary structure analyses and biological assay of the released proteins showed that encapsulation and release did not affect the protein conformation and functionality. The same group also designed amphiphilic peptide to form stable nanoweb and slowly releases encapsulated hydrophobic drug.⁵⁹ In addition, 9-residue peptide N-Pro-Ser-Phe-Cys-Phe-Lys-Glu-Pro-C can effectively stop animal bleeding within 30 s.

Banerjee and co-workers developed supramolecular hydrogel for encapsulation and sustained release of drugs by introducing non proteolytic segment on gelator structure.⁶⁰ Their design was based on the stability of β -amino acid toward enzyme degradation. Two Fmoc-dipeptides contain β -alanine residue were used as gelator and matrix for controlled release of vitamin B₂ and vitamin B₁₂. Their work revealed that diffusion of drugs was

affected by molecule size. The release of vitamin B₂ was faster than vitamin B₁₂ as vitamin B₂ was smaller than vitamin B₁₂. Furthermore, hydrogels with encapsulated drug were stable during over 70 hour observation with the presence of water on top of hydrogels.

Figure 1.18. a) Graphical representation of functional protein for controlled release; b) release profile of functional proteins through self-assembling peptide hydrogel in PBS (pH 7.4) at room temperature. Inset is protein release plotted as function of the square root of time showing a biphasic diffusion mechanism. Reprinted with permission from reference 57. Copyright 2009 Proceedings of the National Academy of Sciences of the United States of America.

Figure 1.19. a) Chemical structure of non proteolytic Fmoc-dipeptide; b) Optical images of diffusion of vitamin B₂ (left) and vitamin B₁₂ from hydrogel; c) Release of vitamin B₂ from hydrogels; d) Release of vitamin B₁₂ from hydrogels. Reprinted with permission from reference 60. Copyright 2012, The Royal Society of Chemistry.

1.3.2 Catalyst

The application of gel as medium for reaction is resulted as a consequence of the appearance of cooperative interactions by proximity of catalytic group. For example, Escuder and co-worker described supramolecular hydrogel based on L-proline derivate as heterogeneous catalyst for aldol reaction between cyclohexanone and 4-nitrobenzaldehyde.⁶¹ Their catalytic experiments shown that diastereoselectivity and stereoselectivity of hydrogel was

improved when the reaction took place at low temperature which related to restricted mobility of the reactants as well as the structural rigidification of supramolecular aggregates which allow a better discrimination of the reaction pathway that lead to the different stereoisomers. Furthermore, hydrogel could be reused for at least two additional run with the same efficiency and stereoselectivity (Figure 1.20). They also provided evidence that the hydrophobic effect was a key role on the mechanism of hydrogel-catalyzed aldol reaction.⁶² The hydrogel shown almost undetected amount of product for short chain keton, moderate yield for ketones with 5 and 6 carbon atoms and increased considerably for longer chains of 2-nonanone and 2-dodecanone.

Another catalytic system based on supramolecular gel was the oxidation of benzyl alcohol into benzyldehyde within pyridine-modified amino acid organogel.⁶³ This catalytic system was based on conventional catalytic activity of Palladium (Pd). Pd-pyridine complexes were formed by diffusion of Pd(Ac)₂ solution into pre-formed organogel (Figure 1.21). Pd-functionalized organogel was able to catalyzed benzyl alcohol oxidation with maximum yield 50% within 48 hours. This type of organogel was different from common organometallogels reported since the coordination between Pd and pyridine occurred after the gelation took place. Control experiment showed that when gelator and Pd salt were mixed there was no gelation took place and the mixture turn black after few minutes.

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Figure 1.21. a) chemical structure of pyridine-based organogelator for oxidation of benzyl alcohol; b) Schematic representation Pd-modified pyridine-based organogel for catalytic oxidation of benzyl alcohol. Reprinted with permission from reference 63. Copyright 2005, The Royal Society of Chemistry.

1.3.3 Electronic Devices

Recently, there was growing interest in development molecular electronic devices, particularly the construction of nanometer scale architecture that has conductive properties.⁶⁴ Using programmed self-assembly, the conductivity could arise from proper arrangement of electro active molecule (chromophores) within gel phase materials. In this way, gel phase facilitated energy transfer process between donor and acceptor molecules. This process of energy transfer was inspired by nature, in which natural pigments (chlorophyl)

in photosynthetic membrane were organized in fix spatial arrangement by surrounding polypeptide with the help of non covalent interaction.^{65,66}

The early example of construction chromophore in gel phase material was work by Sagawa and co-worker. Pyrene functionalized glutamate and porphyrin functionalized glutamate were used as building block for light harvesting gel.⁶⁷ Nakashima and co-workers have established energy transfer from naphthalene derivate to anthracene derivate through electrostatic interaction within cationic L-glutamate hydrogel.⁶⁸

Figure 1.22. a) Molecular structure of L-glutamate derivatives and anionic fluorophores; b) Schematic representation of the hierarchical organization of supramolecular hydrogel. Reprinted with permission from ref 65. Copyright 2002, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

In addition to controlling chromophore in appropriate manner in order to facilitate energy transfer, supramolecular gel also find application in controlling nanostructure of inorganic materials. Hanabusa and co-workers demonstrated that the morphology of TiO_2 can be controlled using organogel-templated synthesis.⁶⁹ Their work are based on two type l-lysine based gelators: uncharged gelator and negatively charge gelator. Uncharged gelator act as template and result in TiO_2 nanotubes, whereas negatively charge gelator did not act as template and resulted nanoparticles. Proposed scheme for organogel-templated synthesis of TiO_2 is depicted on Figure 1.23.

Figure 1.23. Fabrication mechanism of TiO_2 nanotube and TiO_2 nanoparticles in organogels. Reprinted with permission from reference 69. Copyright 2006, The Royal Society of Chemistry.

Utilization of self-assembly peptide nanofibers as template for inorganic synthesis also appeared in fabrication of cathode material for lithium ion batteries. Park and co-worker synthesized iron (III) phosphate using Fmoc-diphenylalanine (Fmoc-FF) hydrogel as template.⁷⁰ Peptide nanofiber was able to adsorb ferric ion and phosphate ion to form mineralized peptide hydrogel. After calcinations, mineralized nanofiber was converted into FePO₄ nanotubes with layer conducting carbon coated the inner surface of nanotube (Figure 1.24).

Figure 1.24. a) Formation of FePO₄ nanotube using Fmoc-FF hydrogel as template; b) Low magnification of SEM image of FePO₄ nanotube formed after calcinations; c) High magnification of SEM images FePO₄ revealed the opening part of nanotube (arrow). Reprinted with permission from reference 70. Copyright 2010, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Combining photo-responsive molecule into supramolecular gel provide unique properties of gel. Banerjee and co-workers synthesized hydrogelator based on amino acid-modified (tyrosine) perylene bisimide.⁷¹ The gelator displayed a pH responsive, semiconducting and photo-responsive behavior. The pH responsive behavior of hydrogel was resulted from amino acid whereas semiconducting and photo-responsive behavior were resulted from π -conjugated system of perylene group. This type of supramolecular hydrogels exhibit high ON/OFF ratio for both UV light and white light (Figure 1.25).

Figure 1.25. Molecular structure of amino acid-modified perylene bisimide gelator; b) I-V curve of device made of gelator shown photoresponsive behavior; c) ON/OFF photocurrent device made of gelator under illumination of visible and white light. Reprinted with permission from reference 71. Copyright 2012, The Royal Society of Chemistry.

Chapter 2 RESEARCH MOTIVATION AND OBJECTIVES

2.1. Motivation and Objectives

The diversity of molecular design for fabrication of supramolecular gel has been described in the previous chapter. However, realizing of simple molecular gelator with advanced functions is continued to challenge. We have already introduced that building block for supramolecular gel required multi steps covalent practices that reduced their practicability. For example, peptide lego developed by Zhang's group at MIT or peptide amphiphile develop by Stupp's group at Northwestern University have at least 16 and 12 amino acid residues respectively. Hence it required high cost, time consuming and skillfull person to synthesis long peptide chain. Furthermore, it seems that one particular system is applicable for only one particular application but either impractical or find no use for other applications. Hence, in my PhD studies I focused on design new structurally simple molecular gelator and explore potential application in wide range applications. Since those oligopeptides are normally synthesized using commercial Fmoc-amino acids as precursor and are taking advantage of intermolecular π - π stacking of aromatic group as well as hydrogen bond in order to form self-assembly structures, it would be convenient and cost-effective if the commercial Fmoc-amino acid or slightly modified Fmoc-amino acids can directly be used as gelator for supramolecular gel. As molecular gelator, Fmoc-amino acids directly provide aromatic structure (Fmoc group)

and hydrogen bond sites (amide bond and carboxylic acid group) which are required to form non covalent interactions.

In order to achieve those objectives, two supramolecular gel systems were prepared: firstly, new supramolecular organogel based on Fmoc-amino acid derivate with easy and simple synthesis was introduced. These thermoresponsive supramolecular gelator was the first reported of Fmoc-amino acid based supramolecular organogel. These supramolecular organogel systems which were synthesized via one step amide coupling were studied using various spectroscopy techniques. Structural design of Fmoc-amino acid derivate as building block for supramolecular organogel offers advantages for various applications. Aromatic moieties promote interaction to aromatic chromophore, whereas amide bond and urethane bond provide the possibility of hydrogen bond interaction. Beside act as super gelator, in which minimum gelation concentration $< 1\%$ (w/v), supramolecular organogel then been investigated for wide range potential application from environmental (dye absorption) to light harvesting gel.

Secondly, a straight forward fabrication of supramolecular hydrogel without relying on time consuming and complicated covalent practices was introduced. The supramolecular hydrogel was based on self-assembly of Fmoc-amino acid. This class of molecules was the shortest molecular gelator based on Fmoc-induced self-assembly into hydrogel. Furthermore, Fmoc-amino acid hydrogel was found as versatile platform for fabrication of antimicrobial hydrogel. Since Fmoc-amino acids are widely used as precursor for oligopeptide synthesis, fabrication of antimicrobial supramolecular hydrogel

from Fmoc-amino acid instead of Fmoc-modified oligopeptide is highly attractive from commercial point of view, being significantly cheaper than Fmoc-modified oligopeptide. In addition, a systematic study of antibacterial behavior revealed that Fmoc-amino acid hydrogels possess fast killing rate and are able to kill bacteria over a wide range of bacteria concentration.

2.2. Organization of the Thesis

The thesis is divided into five chapters. Chapter 1 summarized the comprehensive background and development of supramolecular gel-phase materials including the design and applications. Subsequently, Chapter 2 focused on what motivated us to study and design new supramolecular gel system. Chapter 3 described comprehensively how to fabricate supramolecular organogel based on Fmoc-amino acid derivative and their application. Chapter 4 described water gelation by self-assembly of Fmoc amino acid and their application for antimicrobial hydrogel. Finally the thesis will close with conclusions and recommendations in Chapter 5.

Chapter 3 SUPRAMOLECULAR ORGANOGE

BASED ON FMOC-AMINO ACID DERIVATE

In this chapter, new supramolecular organogel based on Fmoc-amino acid derivate is presented. Molecular gelator was synthesized via one step coupling which is directly converting non gelator Fmoc amino acid, Fmoc-Lys(Boc)-OH into organogelator, Fmoc-Lys(Boc)-C16. A systematic characterization of supramolecular gel was conducted to study the gelation mechanism. Their applications as light harvesting organogel were explored. High efficiency energy transfer (~89%) was achieved between donor (Fmoc-group) and acceptor (dansyl group).

3.1. Introduction

Due to the interesting physicochemical and morphological properties, the self-assembly of low molecular weight organogelators for supramolecular gels currently receives significant interest.^{67,72–82} Gels are constructed by self assembly of small building blocks into fibrous network mediated by weak unidirectional non covalent interaction such as hydrogen bonding,⁷⁹ π - π stacking,⁵³ and van der Waals interactions.⁸³ Since these networks form by weak interactions, the sol and gel phase can be easily transformed by heating and cooling process leading to the thermo based reversibility. Most organogelators

have functional groups, such as carbohydrates,^{84,85} amino acids,⁸⁶⁻⁹² urea,⁹³ and cholesterol,^{94,95} which can undergo non covalent intermolecular interaction.

Despite serendipity still plays an important role in gelators discovery, a number of strategies exist to design molecular gelator which was mainly inspired by natural structural motifs on peptide self-assembly including coiled-coils, β -sheets, and β -hairpins. Zhang and colleagues synthesized alternating charged peptide as building block for peptide hydrogels,² whereas Stupp and co-workers used peptide amphiphile system to fabricate pH sensitive hydrogels.⁹⁶ While many gelators bear hydrophobic alkyl chain and long peptide sequence, only small number of gelators constructed by short peptide sequence and relied on aromatic-aromatic interaction to promote gelation both in aqueous and organic solvent. Ulijn and co-workers established dipeptide hydrogels system by utilized *N*-fluorenylmethoxycarbonyl diphenylalanine (Fmoc-FF) which forms a hydrogel under physiological conditions.⁴⁷ The Fmoc moiety is widely used as a protecting group in solid phase peptide synthesis and it was even reported by Burch et al. that a number of Fmoc-amino acids show anti-inflammatory properties.⁹⁷ After that a number of Fmoc-tri-,tetra-, and penta- peptide-based gelators were examined.^{52,53,98}

While many Fmoc-peptide based gelators have been successfully exploited as building blocks for supramolecular hydrogels, there were limited amount of report available to evaluate Fmoc- moiety ability to form supramolecular gels in organic solvent. Low molecular weight organogelator is important due to their potential application in the field of nanotechnology,⁹⁹ water treatment,^{92,100} oil-spilled solidifiers,¹⁰¹ food and cosmetic technology,¹⁰²

light harvesting materials,^{66,103} pharmaceutical organogels,¹⁰⁴ and sensing.¹⁰⁵ Recently, Cao et.al reported the self-assembly racemic mixture of Fmoc-alanine derivate in hexane and found that the system can be used to determine the enantiomeric in a mixed system.¹⁰⁶

Encouraged by the potential application and promising prospect of supramolecular organogels in recent works, we investigate the supramolecular organogels based on Fmoc-amino acid. Because amino acids are the component for a living body, they are offer biocompatibility and environmental friendly materials. In addition, amino acids are commercially available at relatively low price and the synthetic strategy of their derivatives is established.⁸⁶ In this work, we report a new type of organogelator, based on Fmoc-lys(boc)-c16 which exhibit supergelator properties since it requires ultra low minimum gelation concentration. In addition, we also reported the utilization of Fmoc-lys(boc)-c16 gel as a template for chromophore assembly for efficient light harvesting soft materials. The key feature of our gelator which is constructed from Fmoc-amino acid moiety thus provides an example how simple gelator can be utilized in diverse applications as multi-purposed organogels which have not been demonstrated before.

3.2. Experimental

3.2.1. Materials

Fmoc-lys(boc)-OH was purchased from Merck. N,N-diisopropylethylamine (DIPEA), 5-(Dimethylamino)naphthalene-1-sulfonyl chloride (Dansyl Chloride), Octylamine and Rhodamine-6G were purchased

from Aldrich. Dodecylamine, Hexane, Cyclohexane, Dichloromethane (DCM), Methanol and Ethanol were purchased from Fisher Scientific. O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) was purchased from GL Biochem (Shanghai) Ltd. All chemical reagents were used as received.

3.2.2. General Technique

Infra-red spectra were collected using Perkin Elmer FTIR, UV-Vis spectra were obtained from Shimadzu UV-2501 PC, Fluorescence characterization were conducted using Shimadzu RF-5301 PC, Circular Dichorism spectra were obtained from Jasco J-810 Spectropolarimeter, m/z spectra of compound were collected from Shimadzu AXIMA MALDI TOF2, and high resolution of scanning electron microscopy images were collected from FE-SEM JSM-6340.

3.2.3. Synthesis of Gelator

Fmoc-lys(boc)-C16 were prepared by one step coupling between Fmoc-lys(boc)-OH and Dodecylamine using O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as coupling agent. Briefly, Fmoc-lys(boc)-OH (1 mmol) was dissolved in dichloromethane (DCM) (15mL) in an ice-water bath. Dodecylamine (2 mmol) was added to the reaction mixture, followed immediately by N,N-diisopropylethylamine (DIPEA) (2 mmol) and HBTU (2 mmol). The reaction mixture was allowed to come to room temperature and stirred for 12 h. DCM was evaporated and the precipitated was

washed with ethyl acetate and dried. The crude product was dissolved in 100 mL hot methanol and cooled to room temperature. The gel formed was filtered and dried. The purification was conducted by dissolving the dried product in hot ethanol and cooled to room temperature to get precipitate. This process repeated several time. The product was characterized using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

3.2.4. Gel Preparation

The gelation test were performed by dissolving the required amount of gelator in hexane or cyclohexane in a vial with an internal diameter 10mm by slowly heating and then were allowed to cool slowly (undisturbed) to achieve room temperature. After 20-30 min, gel formation was verified by vial inversion method. Gels were characterized using various spectroscopy techniques including FTIR, Fluorescence as well as Circular Dichroism. Microstructures of dried gel were characterized using field emission SEM.

3.2.5. Energy Transfer Study

Octyldansyl as acceptor was synthesized by coupling between dansyl chloride and octylamine followed the method reported by Summers, et.al.¹⁰⁷ Energy transfer study was conducted by mixed different concentration of octyldansyl into 0.5mL of gelator solution (0.33% w/v in cyclohexane). After the gels were formed, fluorescent spectroscopy measurements were conducted.

3.2.6. Dye Absorption Study

Dye adsorption was conducted by placing 0.5 mL of Rhodamine 6G solution (0.01 mM in 0.1 (v/v %) of TFA solution) on top of 1 % (w/v) of organogel. This test was left for 24 hour at room temperature. Rhodamine 6G left on solution was monitored at time interval 1, 3, 6 and 24 hour by UV-Vis.

3.3. Results and Discussions

3.3.1. Synthesis of Gelator and Organogel Preparation

The coupling of Fmoc-lys(boc)-OH with dodecylamine extremely converted non gelator, Fmoc-lys(boc)-OH into gelator compound (Figure 3.1). MALDI-TOF spectra revealed the presence of molecular gelator as indicated by m/z peaks (Figure 3.2). Fmoc-lys(boc)-C16 formed transparent self-supporting organogels in hexane and cyclohexane with minimum gelation concentration (MGC, w/v) of 0.3 and 0.2, respectively.

Figure 3.1. a) Synthesis of Gelator Fmoc-lys(boc)-C16 via one step coupling between Fmoc-lys(boc)-OH and Dodecylamine; b) Fmoc-lys(boc)-C16 formed

transparent self-supporting gel in hexane, it also possessed phase selective gelation behavior.

Figure 3.2. MALDI-ToF MS of Fmoc-lys(boc)-C16. Peaks at m/z 716 and 732 correspond $[M+Na]^+$ and $[M+K]^+$ respectively.

Interestingly, when the gelator confronted with a biphasic system of water and hexane or cyclohexane, the gelation took place selectively on organic phase which indicated the gelator had phase-selective gelation behavior (Figure 3.1.b). To obtain the morphology of gel in hexane and cyclohexane, the morphologies of gel were characterized by scanning electron microscopy. The morphology of dried gels showed the formation of three-dimensional network structure as a result of anisotropic self-assembling (Figure 3.3).

Figure 3.3. a) FESEM image of Fmoc-lys(boc)-C16 gel in cyclohexane at different magnification; b) FESEM image of Fmoc-lys(boc)-C16 in hexane at different magnification. Both images revealed that organogels were consisted of fibrous structures.

Structurally, Fmoc-lys(boc)-C16 consisted of three functional parts: 1) fluorenyl aromatic moiety; 2) hydrogen bond sites (amide and urethane); and 3) long alkyl chain. In order to understand the mechanism of Fmoc-lys(boc)-C16 self-assembly, both FTIR and fluorescent measurement were conducted. The nature of hydrogen bond of gelators can be easily determined using FTIR, whereas the aromatic interaction of fluorenyl moiety can be evaluated using fluorescence spectra and circular dichroism (CD). According to FTIR spectrum

of Fmoc-lys(boc)-C16, there is no obvious different for C=O urethane and C=O amide of solid (black line) and gel state (red line) of gelator molecules (figure 2a). All the peaks appear around 1650 cm^{-1} for C=O amide, and 1680 cm^{-1} for C=O urethane (Figure 3.4). This fact indicated intermolecular hydrogen bonding of gelator in gel state is close to the solid state, whereas the isotropic solution (blue line) of Fmoc-lys(boc)-C16 in chloroform afford higher peak for both amide and urethane which indicated non-hydrogen bonding.

Figure 3.4. FTIR Spectra of Fmoc-lys(boc)-16 in three different phase. Intermolecular hydrogen bond resulted lower amide peak and urethane peak for gel phase appear at lower wavenumber than solution phase.

To achieve more information regarding Fmoc- group orientation, we conducted fluorescent spectroscopy measurement to study the fluorenyl aggregation in gel phase supplemented with circular dichroism spectroscopy measurement. A weak emission peaks around 319 nm appear in solution state (blue line) whereas in gel states (red line), there is a red shift to 325nm, indicated the anti parallel mode of fluorenyl moiety (Figure 3.5).¹⁰⁸

Figure 3.5. Fluorescence spectra of fluorenyl group in solution and gel phase at excitation wave length 280 nm. A strong peak centered around 325 nm and shoulder around 360 nm indicated the presence of fluorenyl aggregation, whereas solution phase resulted only a weak peak centered around 319 nm.

Moreover, a concentration dependent of CD spectra (Figure 3.6) revealed the formation of fluorenyl moiety aggregation as indicated by red shift

of π - π^* transition of fluorenyl group from 275nm (monomer/solution, blue line) to ~308 nm (self-assembled form/gel state, red line and black line). Furthermore, temperature dependent of CD spectra provide the evidence for thermo-reversible properties of Fmoc-lys(boc)-C16. Upon heating, the intensity of peak at 308 nm started to decrease at 60°C and simultaneously disappeared at 70°C (Figure 3.7). Meanwhile, heating and cooling curve revealed that the nucleation of monomer into fibrous structure started at 40°C (Figure 3.8)

Figure 3.6. Concentration dependent of circular dichroism (CD) spectra of Fmoc-lys(boc)-c16 organogel in cyclohexane

Figure 3.7. Temperature dependent of circular dichroism (CD) spectra of Fmoc-lys(boc)-c16 revealed the disassembly process as indicated by decrease fluorenyl peak as temperature increase.

Figure 3.8. Organogel possessed thermal-reversible behavior as indicated by heating and cooling cycle.

Based on FTIR, Fluorescent spectroscopy and CD spectroscopy we assumed that both hydrogen bond and Fmoc phi-phi stacking are cooperatively driving the self-assembly of Fmoc-lys(boc)-C16 into organogel (Figure 3.9).

Figure 3.9. Proposed molecular arrangement of supramolecular organogel consisted of hydrogen bond and aromatic-aromatic interaction.

3.3.2. Light Harvesting Organogel

Since supramolecular gels were constructed by 1D alignment of gelator arranged via noncovalent interaction at proper distances and in a relevant orientation, supramolecular gels can be used as template for chromophore assembly. In nature, chlorophyll molecules as chromophore for photosynthesis are arranged in continuous unidirectional within peptide matrix and play key role in energy transfer in photosynthetic system. In addition to being integral to gelation, fluorenyl moiety also convenient chromophore with which to probe energy transfer. When the fibre-forming molecules include a suitable donor chromophore, these fibers can be used to transfer energy to a second acceptor chromophore within the system.¹⁰⁹

Since Fmoc-lys(boc)-C16 successfully formed gel in cyclohexane, we then utilized it as medium for chromophore assembly. The system comprised fluorenyl donor and dansyl acceptor. Following the gelation by fluorescence, an immediate excitation at 280 nm to hot solution of gelator results emission of Fmoc group at 330 nm (black line) and when stable transparent gel formed, a decrease in the intensity take place (red line). In addition, the shoulder at 360nm became prone which indicate the formation of π - π stacking of Fmoc group (Figure 3.10). Emission at region 330-360 nm suitable for energy transfer to dansyl group since there is overlapping between Fmoc emission and excitation maximum of dansyl which centered at around 350 nm (green line). The addition of acceptor group (dansyl) decrease emission of Fmoc group (blue line). The intensity of emission at region 330-360 nm was lower than in the absence of dansyl, indicating that energy transfer occurs between Fmoc and dansyl.

Figure 3.10. Energy transfer between fluorenyl group and dansyl group. Excitation spectra of dansyl overlapped with emission spectra of fluorenyl.

Figure 3.11. Schematic representation of energy transfer between fluorenyl group and dansyl group within gel phase.

In order to investigate the efficiency of energy transfer, we conducted a concentration dependent study at MGC of gelator. Energy transfer efficiency was calculated from the quenching effect of dansyl for Fmoc-lys(boc)-C16 at the emission maximum at 330 nm. Figure 3.12 depicted concentration dependent of energy transfer at various concentration of dansyl. It was found that the emission intensity of Fmoc group decreased as the concentration of dansyl was increased. It was also revealed that maximum quenching of Fmoc emission take place at 8.28×10^{-4} M of dansyl with efficiency $\sim 90\%$. Moreover, the energy transfer between fluorenyl-dansyl system shown high efficiency as the inclusion of 3% mol (1.65×10^{-4} M) of acceptor caused a 50% quenching of the fluorenyl emission. This case much more ideal for light-harvesting system compare to oligo(p-phenylenevinylene)- (OPV-)based organogels,¹¹⁰ in which a 5-fold excess of acceptor was necessary for essential quenching of donor.

Figure 3.12. Concentration dependent of energy transfer within gel phase.

Increasing the dansyl concentration simultaneously decrease the emission spectra of fluorenyl at 330 nm.

3.3.3. Dye Adsorption

Rhodamine-6G adsorption on Fmoc-lys(boc)-C16 organogel was depicted in Figure 3.13. As we can see, it was only take 6 hours for organogel to adsorb more than 80% rhodamine-6g from solution. It was also revealed that maximum adsorption of rhodamine-6g was achieved after 24 hour with ~93% adsorption.

The ability of Fmoc-lys(boc)-C16 to adsorb rhodamine-6g was resulted from diffusion of rhodamine-6g into gel phase. The presence of aromatic structure and hydrogen bonding sites promote diffusion of rhodamine-6g into

gel phase. Our control experiment revealed that the higher gelator concentration, the better adsorption properties of organogel. Figure 3.14 depicted effect of gelator concentration on rhodamine-6g adsorption. It revealed that gel with 1% gelator adsorb almost two times faster than 0.5% gelator. Morphology study of dried gel after adsorption revealed that the fibrous structures were preserved during rhodamine-6g adsorption.

Figure 3.13. Time-dependent curve of percentage of rhodamine-6g adsorption on organogel. Inset figure: Uv-vis spectra of rhodamine-6g aliquot at different time after contact with organogels.

Figure 3.14. UV-Vis spectra of rhodamine-6g after contact with different concentration of organogel.

Figure 3.15. Photograph of rhodamine-6g adsorption on Fmoc-lys(boc)-C16 Organogel (left). Image on right side depicted the scanning electron microscope of dried organogel after rhodamine-6g adsorption.

3.4. Conclusion

In this chapter, we have demonstrated that simple low molecular weight organogelator based on Fmoc-amino acid derivate can be applied for multi-purposed supramolecular gels. The formation of the organogels was well characterized using different spectroscopy method. We notice that cooperative H-bond and π - π stacking are responsible for Fmoc-lys(boc)-C16 gel self-assembly. Beside its potential application in phase-selective gelation, the stable and transparent gel also can be used for chromophore assembly for light harvesting soft materials. We realized high efficiency energy transfer (~89%) between fluorenyl and dansyl in gel phase. Furthermore, as multi-purposed organogel, we also explored potential application of Fmoc-lys(boc)-C16 organogel as organic pollutant removal. Our study revealed that Fmoc-lys(boc)-C16 exhibited efficient rhodamine-6g adsorption.

Chapter 4 SUPRAMOLECULAR ANTIBACTERIAL HYDROGEL BASED ON SELF-ASSEMBLY OF FMOC-AMINO ACID

This chapter described the formation of supramolecular hydrogel via simple and facile approach using Fmoc-amino acid as building block. Fmoc-amino acid hydrogel was subsequently used to fabricate antimicrobial supramolecular hydrogel without involving time consuming covalent practices.

4.1. Introduction

The development of supramolecular hydrogels as new approach for combating global spreads of drug-resistance microbial infection associated with wound healing and medical devices have attracted increasing attention in the field of biomedicine.^{111–114} Due to high water content, supramolecular hydrogel provide a moist, heavily hydrated environment to the wound area, facilitating cellular activity which essential to the wound healing process and at the same time exhibit antimicrobial activity.¹¹⁵ Most of the current work in antimicrobial supramolecular hydrogel focuses on the supramolecular assembly of synthetic cationic polymer and cationic antimicrobial peptide (AMP).^{116–119} Nevertheless, the preparation of cationic polymer and antimicrobial peptide requires multiple steps and complicated chemical synthesis and purification, which may affect the quality of hydrogel as well as antimicrobial efficacy. It has been reported that antimicrobial activity of cationic polymer were strongly affected by

molecular weight, cross-linker agent as well as spacer length and alkyl chain of polymer.¹²⁰ Moreover, organic solvent and toxic chemicals used in such covalent practices could be embedded in polymer and antimicrobial peptide and reduce their biocompatibility.¹²¹ In addition, the application of cationic materials as antimicrobial hydrogel may cause major concern of hemolysis and most of polymeric-based antimicrobial reported in the literature are suffering from immunogenicity as well as inflammation.^{122,123} Therefore, it is necessary and beneficial to develop a simple, cost effective and biocompatible antimicrobial supramolecular hydrogels.

In the past few years, supramolecular hydrogels derived from low molecular weight gelator based on 9-fluorenylmethoxycarbonyl-modified oligopeptide (Fmoc-oligopeptide) have received great interest due to the biocompatibility in various biomedical applications including drug delivery as well as 2D and 3D cell culture.^{124–126} The gelator self-assembled in aqueous solvent to give long fibrillar supramolecular structures which entangle to form a 3D structure driven by non-covalent forces such as hydrogen bonds, π - π interactions, electrostatic interactions as well as van der Waals interactions. As emerging class of self-assembling systems, a number of supramolecular hydrogels based on Fmoc-modified oligopeptide which can be prepared by enzymatic, temperature, solvent and pH trigger have been reported.^{47,50–53,98,127–129} Although those studies described the biocompatibility of Fmoc-modified oligopeptide supramolecular hydrogel, only few reports described the utilization of Fmoc-modified oligopeptide as platform for antimicrobial supramolecular hydrogel.¹³⁰ Nevertheless, those Fmoc-modified oligopeptide

required further chemical functionalization in order to be used as potent antimicrobial agent and hence possessed the same challenge with polymer and peptide-based antimicrobial.

In this chapter, we report a novel strategy for the fabrication of antimicrobial supramolecular hydrogel by using co-assembly of Fmoc-amino acids as building block for supramolecular hydrogel. For the point of antimicrobial ability conferment, co-assembly is a valuable strategy which is always used in supramolecular systems to confer additional properties for different applications.^{131–134} Since Fmoc-amino acids are widely used as precursor for oligopeptide synthesis, fabrication of antimicrobial supramolecular hydrogel from Fmoc-amino acid instead of Fmoc-modified oligopeptide is highly attractive from commercial point of view, being significantly cheaper than Fmoc-modified oligopeptide. Thus, taking advantages of Fmoc-assisted self-assembly of small molecule (amino acid) into hydrogel as well as the ability of hydrogel to encapsulate bioactive compound, a facile route for antimicrobial hydrogel can be realized without involving expensive and time consuming covalent practices. As proof of concept, we use Fmoc-phenylalanine (F) and Fmoc-leucine (L) as building block for supramolecular hydrogel and antimicrobial agent respectively. Peptide with leucine-rich residues has been known for their bacteria recognition and elimination.^{135–138} Additionally, L has also been reported to possess intrinsic anti-inflammatory properties.^{97,139} Therefore, we hypothesize that by combining commercial Fmoc-leucine (L) and Fmoc-phenylalanine (F) together used as building block of antimicrobial agent and supramolecular hydrogel

respectively, a co-assembly strategy for the preparation of biocompatible antimicrobial hydrogel could be realized. This strategy for antimicrobial supramolecular hydrogel which relying on the co-assembly of commercially available Fmoc amino acids will surely enabled all of us to fabricate biocompatible antimicrobial hydrogels easily and cost-effectively without involving complicated organic synthesis and purification processes, compared to commonly used antimicrobial hydrogels based on cationic materials. In this case, Fmoc-amino acid acts as ideal building blocks for fabrication of supramolecular self-assembly-based new technologies since it provides an exceptionally simple fabrication and molecule design as well as easy tailored for diverse applications.¹⁴⁰

4.2. Experimental

4.2.1. Materials

Fmoc-glycine, Fmoc-alanine, Fmoc-valine, Fmoc-leucine, Fmoc-isoleucine and Fmoc-phenylalanine were purchased from Merck and used as received. Sodium carbonate and Luria-Bertani broth medium were purchased from Sigma-Aldrich. SYTO 9 and propidium iodide fluorescent dye were purchased from Invitrogen.

4.2.2. Hydrogel Preparation

The gelation test were performed by dissolved 10 mg of Fmoc-amino acid in a vial with an internal diameter 10 mm containing 500 μ L of water and equivalent amount of sodium carbonate followed by slowly heating until all solid disappear. The hot solutions were allowed to cool slowly (undisturbed) to

achieve room temperature. After 20-30 min, gel formation was verified by vial inversion method. (F+L) hydrogel were prepared in an identical manner but contain 2 mg of Fmoc-leucine and 10 mg of Fmoc-phenylalanine. The morphology and the microstructures of dried gel were characterized by field Emission (FE) SEM (JSM-7600F from JEOL). Fluorescence emission spectra were recorded on a spectrofluorophotometer (RF-5301 PC). The Raman spectra were obtained with a spectrophotometer (WITec alpha 300 R). The operating wavelength was 532 nm. FTIR spectra were recorded on a FTIR system (PerkinElmer).

4.2.3. Molecular Dynamic Simulation

To investigate the gel formation, the six kinds of Fmoc-amino acid (125 molecules) were random put into water box (4494 molecules) respectively, and then the self-assembly processes began from a random initial configuration with atomic MD simulations. The GROMACS 4.05 simulation package and GROMOS96 force field were used for all our MD simulations.^{141,142} The six types of Fmoc molecular models employed in this study were generated from the small-molecule topology generator PRODRG. The water was modeled using the single point charge (SPC) model, with the bond lengths and angles held constant through the use of the SETTLE algorithm. Bond lengths of dendrimers were constrained using the LINCS algorithm.

Simulations were performed using the NPT ensemble. In this ensemble, the number of molecules (N), the pressure (P), and the temperature (T) are fixed. The temperature was maintained at 300 K using the Berendsen temperature coupling method and Berendsen bath coupling scheme was used to

keep a constant normal pressure of 1 bar.¹⁴³ The cutoff distance for short-range nonbonded interactions was chosen to be 12 Å and long-range electrostatic forces were computed using the Reaction-Field (Particle Mesh Ewald) approach.^{144,145} A time step of 2 fs was employed. All the simulations were 2 ns long, and the last 1 ns trajectory was used for analysis, during which the potential energy, the dimensions of the simulation box and interfacial tension remained stable. The picture prepared with VMD.¹⁴⁶

4.2.4. Bacteria culture

Bacteria of *Staphylococcus aureus* (*S. aureus*) (ATCC 6538) were used in our experiments. Prior to experiments, the bacteria were grown overnight in Luria-Bertani broth medium (LB, Sigma-Aldrich) and harvested at the exponential growth phase via centrifugation. The supernatant was then discarded and the cell pellet was resuspended in PBS. The bacteria concentration could be monitored photometrically by measuring the optical density (OD) at a wavelength of 600 nm. Before performing antibacterial experiments of hydrogels, the OD₆₀₀ values of bacteria stock solutions were re-adjusted to 0.1, which corresponded to the concentrations of 4×10^8 CFU/mL, obtained based on colony counting method.

4.2.5. Time-dependent and concentration-dependent antibacterial experiments of hydrogels

Hydrogels for the antibacterial assays were prepared in 24-well tissue culture plates (NUNC). For time-dependent antibacterial assay, 1 mL of *S. aureus* bacteria LB solution with 1×10^7 CFU/mL was introduced onto the surface of the 2% (w/v) hydrogel (F gel or F+L gel) for 10 h, and the optical

density readings of bacteria solutions were monitored every 2 h by measuring the OD₆₀₀. The bacteria solution without hydrogel or with L solution was used as control. For concentration-dependent antibacterial assay, 1 mL of *S. aureus* LB solution with different bacteria concentration (1×10^4 - 1×10^9 CFU/mL) was introduced onto the surface of the 2% (w/v) hydrogel (F+L gel) for 48 h, and the optical density readings of bacteria solutions were monitored by measuring the OD₆₀₀. The bacteria solution without hydrogel was used as control.

4.2.6. Morphology study of bacteria

The morphology of bacteria before or after hydrogels treatment was examined by a field emission scanning electron microscopy (FESEM). Firstly, the *S. aureus* bacteria solution before or after hydrogels treatment was dropped onto silicon wafer, and the silicon wafer was then treated with 2% glutaraldehyde (Sigma-Aldrich) fixation for 2 h at room temperature and gradient dehydration by a series of ethanol solutions (50%, 70%, 90%, and 100%) for 10 min with each step. Next, the silicon wafer was subjected to nitrogen drying to maintain the morphologies of the bacteria captured on substrate. After complete drying, the silicon wafer was sputter coated with platinum and imaged with FESEM (JSM-6700F, JEOL).

4.2.7. LIVE/DEAD bacterial assay

LIVE/DEAD bacterial assay was carried out to examine the viability of bacteria after hydrogel treatment. After incubated with hydrogel for different times, *S. aureus* bacteria solution was mixed with 0.5 mL of dye solution containing 1 μ M SYTO 9 and 5 μ M propidium iodide (Invitrogen) for 20 min at room temperature. The green colored SYTO 9 dye enters both intact and

membrane-compromised bacterial cells, while the red color propidium iodide dye can only enter membrane-damaged cells. The bacteria were then imaged using a confocal microscopy (TCS SP5, Leica).

4.3. Results and Discussion

4.3.1. Fmoc-amino acid Supramolecular Hydrogel

The general scheme for preparation of transparent and self-supporting F hydrogel is shown in Figure 4.1. Microscopy studies were conducted to observe the morphology of hydrogel. Optical microscopy images reveal the formation micron size long fiber (Figure 4.2). Atomic force microscopy (AFM) analysis of hydrogel shown that the gel were comprised entanglement of fibrils whereas high resolution images of field emission scanning electron microscopy (FESEM) revealed that gel composed by fine fibers with dimension less than 100 nm in width (Figure 4.3.a).

Figure 4.1. The schematic representation of preparation Fmoc-phenylalanine hydrogel.

Figure 4.2. Microscopy images of Fmoc-phenylalanine hydrogel revealed the formation of micron size long fibril structures.

The viscoelastic behavior of Fmoc-phenylalanine gels were determined using rheology measurement. As shown in Figure 4.3.b, storage modulus (G') of hydrogel is approximately an order of magnitude greater than loss modulus (G''), indicating that this gel behave as elastic solid rather than viscous material. Fluorescence spectroscopy measurements were conducted to investigate the molecular arrangement of Fmoc groups in the hydrogel. An emission peak centered at 309 nm appears in solution phase whereas the maximum emission for gel phase appears at 314 nm with the presence of shoulder at 365 nm (Figure 4.3.c). This information clearly indicated the presence of Fmoc aggregation with the existence of antiparallel aromatic-aromatic interaction of Fmoc group in gel phase. It has been proposed that the red shift is possibly due to the formation of Fmoc eximers.¹⁴⁷ To study the nature of hydrogen bond in the self-assembled and non-self-assembled state, FTIR measurements were

conducted. FTIR spectra of hydrogel was characterized by C=O stretching band centered at 1640 cm^{-1} , whereas the isotropic solution of gelator in chloroform afford higher peak, 1658 cm^{-1} , which indicated the absence of intermolecular hydrogen bonding motifs (Figure 4.3.d). Raman spectra provide complementary information, a strong band of C=O stretching is found at 1610 cm^{-1} for gel phase whereas little red shift is found in solution phase, 1613 cm^{-1} (Figure 4.3.e).

Figure 4.3. Photograph of transparent self-supporting hydrogel formed from Fmoc-phenylalanine (left) consist of long fibrous structure as revealed by AFM (centre) and having dimension less than 100 nm as shown by scanning electron microscopic (SEM) image (right); b) Mechanical properties of hydrogel revealed a solid-like gel behavior as indicated by rheology measurement in which storage modulus (G') always higher than loss modulus (G''); c) Fluorescent spectroscopy revealed the presence of aromatic stacking as indicated by red shift of Fmoc emission spectra and shoulder around 365nm; d) FTIR measurement shown the presence of intramolecular hydrogen bond as indicated by shift of C=O stretching peak; e) Raman spectra confirmed the presence of hydrogen bond in hydrogel as indicated by blue shift of C=O stretching band around 1600 cm^{-1} .

According to these spectroscopic measurements we can conclude that aromatic-aromatic interaction and hydrogen bonds are synergistically driving the self-assembly process into long fibrous structure. Note that the presence of aromatic side chain in phenylalanine play key role to stabilize the self-assembly structure by providing an extra aromatic-aromatic interactions which are unable to be provided by other amino acids tested (Figure 4.4 and Figure 4.5)

Figure 4.4. Proposed molecular arrangement of Fmoc-phenylalanine in self-assembly structure which is driven by π - π stacking of Fmoc group and H-bond ($\text{C}=\text{O}\cdots\text{H}-\text{N}$ and $\text{C}=\text{O}\cdots\text{H}-\text{O}$), the intermolecular interaction is stabilized by the presence of phenyl group stacking.

Figure 4.5. Photograph of tested Fmoc amino acid shows no gel formation. a) Fmoc-glycine, b) Fmoc-alanine, c) Fmoc-valine, d) Fmoc-leucine e) Fmoc-isoleucine.

To investigate the unique properties of Fmoc-phenylalanine compare to other Fmoc-amino acids tested, we conducted a molecular dynamic (MD) simulation. Our results confirmed the formation of a three dimensional fibrous network that responsible for gelation in Fmoc-phenylalanine hydrogel, whereas other Fmoc-amino acids resulted a non fibrous aggregation (Figure 4.6 and Figure 4.7).

Figure 4.6. Molecular Dynamics (MD) simulation of Fmoc-phenylalanine (a) shows the formation of fibrous network whereas Fmoc-alanine shown only non fibrous aggregation (b). Images on the right side are mapping of favourite distribution of neighbouring Fmoc-plane, the x axis is distance of two Fmoc planes, from 0.4 nm to 0.6 nm, whereas y axis is cosine of angle between two Fmoc planes, from 0 to 1.

Figure 4.7. Molecular Dynamic Simulation of Fmoc-glycine (a), Fmoc-valine (b), Fmoc-leucine (c) and Fmoc-isoleucine (d) show the formation non fibrous aggregation (left images). Images on the right side are mapping of favourite

distribution of neighbouring Fmoc-plane, the x axis is distance of two Fmoc planes, from 0.4 nm to 0.6 nm, whereas y axis is cosine of angle between two Fmoc planes, from 0 to 1.

4.3.2. Antimicrobial Supramolecular Hydrogel

To utilize Fmoc-phenylalanine hydrogel as platform for antibacterial hydrogel, Fmoc-leucine (L) was incorporated into hydrogel to produce co-assembly (F+L) hydrogel (Figure 4.8.a and Figure 4.8.b). In order to form hydrogel, the mole ratio of gelator F to L should be maintained below 2:1. Above the ratio 2:1, the mixtures failed to form self supporting hydrogels. This result was probably caused by lack of phenyl groups of gelator (F) which are required to stabilize self-assembly structure.

Figure 4.8. Schematic preparation of antibacterial hydrogel using co-assembly of Fmoc-leucine (L) and Fmoc-phenylalanine (F). b) Photograph of self-supporting antibacterial hydrogel. c) Molecular dynamic simulation of the

aggregations of the complex of Fmoc-phenylalanine (F, blue color) and Fmoc-leucine (L, red color) in water.

Figure 4.9. a) Field emission scanning electron microscopy (FESEM) images of the co-assembled (F+L) hydrogel indicated the formation of fibrous structure; b) Oscillatory rheology of the co-assembled (F+L) hydrogel.

The formation of entangled micron size long fibers within antimicrobial gels was found based on scanning electron microscopy technique (Figure 4.9.a). The viscoelastic behavior of antimicrobial hydrogels were determined using rheology measurement. As indicated in Figure 4.9.b, the oscillatory rheology confirmed the formation of a solid like gel material in which G' larger than G'' .

Figure 4.10. a) Fluorescence spectra of the complex of Fmoc-phenylalanine (F) and Fmoc-leucine (L) in solution and gel phase; b) FTIR Spectra of the complex of F and L in solution and gel phase; c) Raman Spectra of the complex of F and L in solution and gel phase.

For Fmoc-oligopeptides hydrogels, intermolecular interactions of π - π stacking of Fmoc groups and hydrogen bonding were reported to play important roles in gel formation.^{47,50,124,125,148,149} Hence, fluorescence spectroscopy was first used to investigate the molecular arrangement of Fmoc groups within our hydrogels. An emission peak centered at 312 nm wavelength appeared in complex of Fmoc-phenylalanine and Fmoc-leucine solution phase, while the maximum emission for co-assembled (F+L) gel appeared at 316 nm with the presence of a shoulder at 368 nm (Figure 4.10.a). This indicated the existence of anti-parallel intermolecular π - π interactions of Fmoc groups in the gel phase.⁴⁷ The phenomenon of red shift of emission peak was also found for Fmoc-phenylalanine (F) gel system. In order to study the role of intermolecular hydrogen bonding in the formation of our hydrogels, fourier transform infrared (FTIR) spectroscopy was subsequently employed. As shown in Figure 4.10.b, compared to the FTIR spectrum in solution phase, the characteristic C=O stretching band peaks in co-assemble (F+L) gel phases blue shifted from 1717 cm^{-1} to 1636 cm^{-1} , indicating the presence of intermolecular hydrogen bonding motifs. Raman spectroscopy provided the consistent information in which the peaks of C=O stretching band in gel phases exhibited blue shift compared to that in solution phases (Figure 4.10.c). According to these spectroscopic results, it can be concluded that both the intermolecular interactions of π - π stacking of Fmoc groups and hydrogen bonding play important roles in the co-assembled (F+L) hydrogel.

Compared to Fmoc-acids without aromatic phenyl group (Fmoc-G, Fmoc-I, Fmoc-V, Fmoc-A, Fmoc-L), only the Fmoc-F could form hydrogel in

our experiments, indicating that the phenyl group of Fmoc-F also plays a critical role in gel formation in addition to the intermolecular π - π stacking interactions of Fmoc groups and hydrogen bonding. The three forces work together to drive the co-assembly of F and L and finally result in hydrogel formation (Figure 4.11).

Figure 4.11. Proposed molecular arrangement of F and L within the co-assembled (F+L) hydrogel. The co-assembly of Fmoc-F and Fmoc-L is driven by the intermolecular π - π stacking interactions of phenyl and Fmoc group, and intermolecular hydrogen bond ($C=O \cdots H-N$ and $C=O \cdots H-O$), (Carbon, nitrogen, oxygen and hydrogen atoms were indicated with cyan, blue, red and white color respectively).

Gel antibacterial properties were evaluated against *S. aureus* which is common nosocomial pathogen and has been reported to cause almost 19,000 deaths annually in the United States.^{150–152} To demonstrate antibacterial properties of hydrogel, 2% (w/v) hydrogel were challenged with 1×10^6 colony forming unit (CFU)/mL of *S. aureus* at various time exposures (Figure 4.12). As shown, the presence of hydrogel completely suppressed bacterial growth

whereas sample without hydrogel shown an increasing number of CFU during the incubation. Additionally, separate control experiment by using F hydrogel shown no indication of antibacterial activity whereas L solution was truly found to inhibit bacterial growth moderately, indicating its intrinsic antibacterial nature. The hydrogel is found effective to inhibit ~95% of bacteria proliferation after 20 h incubation (Figure 4.14.a). The photographs of *S. aureus* agar plates with or without the co-assembled (F+L) hydrogel after 20 h incubation were given (Figure 4.14.b).

To assess the capacity of antibacterial properties of hydrogel, we further challenged the hydrogel with an increasing number of CFU of *S. aureus* and allowed to incubate for 48 h (Figure 4.13). The data shown that hydrogel is capable of inhibiting *S. aureus* proliferation when up to 1×10^8 CFU/mL introduced. When 1×10^9 CFU/mL is introduced, the hydrogel loses its inhibitory capacity. In contrast, control experiment shown that bacteria is able to proliferate even with the lowest number of bacteria.

Figure 4.12. Time-dependent antimicrobial test shows the growth inhibition activity toward *S. aureus*.

Figure 4.13. Concentration-dependent antimicrobial test shows growth inhibition capacity up to 10⁸ CFU/mL.

Figure 4.14. a) Bacteria inhibiting efficiency of hydrogels shown that it can inhibit ~95% *S. aureus* proliferation; b) Photograph of *S. aureus* incubate in agar plate shown no bacteria film formed in the sample containing hydrogel.

To gain insight into the antimicrobial mechanism of hydrogel, we investigated the live/dead cell assay and the morphology of *S. aureus* after incubation. Figure 4.15 shows live/dead staining of *S.aureus* before and after contact with hydrogel. It is clear that, bacteria were killed after contact with

hydrogel as indicated by red color of fluorescent dye. These assays also suggest that bacteria were killed by cell membrane disruption since propidium iodide (red fluorescence dye) is able to penetrate the membrane of dead cell. This finding was supported by FESEM morphological change. Figure 4.16 shows the morphology of *S.aureus* after contact with hydrogels. It clearly shows that treated bacteria undergo cellular deformation. Additionally, debris of lysed cells was also observed. Time-dependent live/dead assay further confirmed that bacteria were killed rapidly within 30 minutes (Figure 4.17). Since (F+L) gel has no positive charge in the structure, the binding interaction between hydrogel and *S. aureus* were unlikely caused by electrostatic interaction. The interaction between *S. aureus* cell wall and hydrogel were suspected to result of synergetic effect of leucine and phenylalanine hydrophobicity. It has been reported that binding to bacteria cell wall and hemolytic activity increase with the increasing hydrophobicity of amino acid.¹⁵³ Furthermore, it has been found that leucine-rich peptide possessed antibacterial potency and peptide having FL sequences were strongly bind to *S. aureus* cell wall.^{135,137}

Figure 4.15. Overlay live (green color) and dead (red color) fluorescence staining of *S. aureus* before (a) and after incubation (b) shown that *S. aureus* undergo cell death via membrane disruption.

Figure 4.16. a) Scanning electron microscopic (SEM) image of *S. aureus* before incubation; b) low magnification of SEM images of *S. aureus* after

incubation; c) high magnification SEM images further confirm the cell wall disruption on *S. aureus*.

Figure 4.17. Time-dependent live/dead cell assays of antibacteria shown that *S. aureus* was killed within 30 minutes. Green color indicated live cell, whereas red color indicated dead cell.

The biocompatibility of the co-assembled (F+L) hydrogel was investigated. As shown in Figure 4.18.a, endothelial cells attached on the co-assembled (F+L) gel adopted healthy spread morphologies and proliferated to near confluency after 24 h incubation, indicating the biocompatibility of the co-assembled antimicrobial (F+L) hydrogel toward normal mammalian cells. The difference of antimicrobial activity of the co-assembled (F+L) hydrogel for gram-positive and gram-negative bacteria was also investigated systematically.

As shown in Figure 4.18.b, the co-assembled (F+L) hydrogel indeed exhibited the selectivity of antimicrobial effect for gram-positive bacteria. Such a phenomenon may relate to the Fmoc-leucine used since leucine-rich repeat (LRR) peptide sequences have been found to recognize the cell wall of gram-positive bacteria rapidly and efficiently rather than the surface of gram-negative bacteria,^{154–157} making subsequent bactericidal action more easily.

Figure 4.18. a) Microscopy image of endothelial cells attached on the co-assembled (F+L) hydrogel-coated surface after 24 h incubation indicates the

biocompatibility of the co-assembled antimicrobial (F+L) hydrogel toward normal mammalian cells; b) The difference of antimicrobial effects of the co-assembled (F+L) hydrogel for gram-positive and gram-negative bacteria. The y axis indicates the increased folds of OD values of the bacteria solution after incubate with the gel for 20 h. Gram-positive bacteria of *Bacillus subtilis* (*B. subtilis*) and *S. aureus*, and gram-negative bacteria of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *E. coli* were tested.

4.4. Conclusions

In conclusion we have demonstrated that Fmoc-amino acids can be used as facile platform for fabrication of supramolecular hydrogel. Our microscopy and spectroscopy measurement revealed that Fmoc-phenylalanine self-assembled into fibrous structure which were drove by aromatic stacking and hydrogen bonds. Moreover, we also provided a straight forward strategy for fabricating supramolecular antimicrobial hydrogel without involving time-consuming covalent practices by exploiting the potency of Fmoc-leucine as bioactive agent. Our results suggest that the hydrogel possesses high and fast antimicrobial activity toward *S. aureus*. This work opens an opportunity for treatment of gram positive bacteria infection which exhibits high prevalent in recent years,¹⁵⁸ presenting great potential as antimicrobial coating in clinical antibacterial applications. In addition, the Fmoc-phenylalanine hydrogelator introduced in this work provides an ideal platform for hydrogel-based research in which gel activity can be easily tailored according to different applications.

Chapter 5 CONCLUSIONS AND RECOMENDATIONS

5.1. Conclusions

In this thesis we aim to fabricate an advanced function of supramolecular gel by facile and simple method. We successfully demonstrated two strategies to achieve those purposes. First, we have successfully fabricated multi-purposed supramolecular organogel which can be used as a light harvesting system with high efficiency, phase selective gel as well as dye removal system. Second, we demonstrated that supramolecular hydrogel for antibacterial purposes could be realized without involving complicated covalent practices via Co-Assembly of Fmoc-amino acid into hydrogel. In this strategy, we found that the Fmoc-phenylalanine (F) could be used as a new hydrogelator, and antimicrobial hydrogel could be easily prepared via co-assembly mechanism by combining this hydrogelator with another commercial Fmoc-leucine (L) as antimicrobial building block. The co-assembled (F+L) hydrogel exhibited efficient antimicrobial effect for gram-positive bacteria, presenting great potential as antimicrobial coating in clinical antibacterial applications.

5.2. Recommendations

The ability of Fmoc-lys(boc)-C16 gelate hexane and cyclohexane inspired us to investigate the potential application of molecular gels to gelate bioactive volatile liquid organic compound and apply it for molecular reservoir-type for sustained release device. A potentially important sustained release

device is for controlling 2-heptanone release in honeybee industry. 2-heptanone is a volatile liquid pheromone produced by honeybees to control parasitic mites, *Varroa destructor* that destroys honey bee colonies.¹⁵⁹ Numerous polymer-based systems for 2-heptanone have been developed.^{160,161} However those systems involve multi-step preparation, not readily biodegradable and low loading of volatile organic compound. In contrast with polymeric system, molecular gelators have no issue for biodegradability since it been made by bio-based and highly compatible material – amino acid. It also exhibit high loading capacity since the major part in organogels is solvent and percentage of gelator normally in the range of 1-5% w/v. In our system, 2-heptanone-containing gel shown a stable self supporting gel. The release of 2-heptanone from gel containing 5% (w/v) of gelator shown slow evaporation rate (Fig. 5.1). During 14 days observation, only less than 12% weight loss of solvent in gel state took place, whereas more than 90% weight loss took place for solution state during 3 days. Meanwhile, scanning electron microscopy images also revealed the nanostructure of 2-heptanone containing gels consist of entangled fibrous networks (Figure 5.2). The slow rate evaporation of solvent in gel state indicated the solvent molecules are immobilized by surface tension that converting the liquid into a coherent gel. Since the solid-like appearance of a gel produce strong interaction between solvent and gelator as the liquid (solvent) is entrapped in solid 3D matrix, the vapor pressure of volatile solvent is lower than their vapor pressure in liquid state. Hence evaporation rate in gel state is slower than their liquid counterpart.

Figure 5.1. Percentage weight loss over time of solvent (2-heptanone) in gel and solution

Figure 5.2. Optical image of 2-heptanone-containing gels (Gelator at 5% w/v) (left), SEM images of 2-heptanone-containing gels at different magnification (middle and right).

Despite we have proved that Fmoc-lys(boc)-C16 is able to gelate 2-heptanone, more details study regarding kinetics and control release (concentration dependent) study is needed so that one is able to provide parameter for the application of supramolecular gel for volatile pheromone release. Furthermore, direct application of supramolecular organogel for controlling *varroa destructor* in honey bee industry should be done.

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List of Publication

Papers

- Irwansyah; Li, Y.-Q.; Shi, W.; Qi, D.; Leow, W. R.; Tang, M. B. Y.; Li, S.; Chen, X. “*Gram-Positive Antimicrobial Activity of Amino Acid-Based Hydrogel*”, *Adv.Mater.***2014**,26, doi:10.1002/adma.201403339
- Irwansyah, Jianjun Du, Yinghui Sun, Xiaodong Chen, “*The Self-assembly of Fmoc-amino Acid Derivate for Multi-purposed Supramolecular Organogels*”, ***In Preparation.***

Poster

- Irwansyah, Sun Yinghui, Du Jianjun, Chen Xiaodong, “*Energy Transfer in Fmoc-amino acid-based Supramolecular Organogels*” **Small Sciences Symposium, 2012, Singapore**