ADVANCED IN SITU HYDROGEL ASSEMBLY FOR GUIDING MOLECULAR RELEASE

BY

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DISSERTATION

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ABSTRACT

Since the emergence of hydrogels as carriers for cells, bioactive molecules, and even metallic nanoparticles, there were extensive efforts to control the rate and direction of embedded molecular release, largely by additional chemical modification of gel-forming polymers. However, these approaches often encountered several challenges including the instability of molecular cargos, the extensive labor of synthesis and purification, and the uncontrollability of the molecular release direction. In contrast, many biological systems use their geometry to guide the release of their molecules or signals. Inspired by nature, this study presents unique approaches with advanced in situ formation techniques, which can overcome the problems and control the release direction and rate of the diverse embedded materials in a hydrogel. First, I demonstrated a self-folding, multi-walled poly(ethylene glycol) diacrylate (PEGDA) hydrogel tube. This tubular structure was obtained by in situ self-folding of a bi-layered PEGDA hydrogel patch constructed with gels of significantly different rigidity and expansion ratio. The radiiuses of the resulting gel tubes were estimated with bilayer curvature equations and agreed with experimental data. Second, the resulting hydrogel was used to control the release rate and direction of embedded molecules by localizing the molecules in a center of the tube. A finite element method (FEM) based simulation was performed to explain the geometrical effect on controlling the molecular release. Additionally, the bilayered PEGDA hydrogel encapsulating VEGF was implanted on a chicken chorioallantoic membrane (CAM) to evaluate the neovascularization. Due to the spatiotemporal release of VEGF, the gel tubes significantly increased the density and diameters of blood vessels, compared to unfolded hydrogel patches and other ring-shaped hydrogels. Third, I presented a bio patch delivery system with minimal invasive manner by using the self-folding and unfolding technique. I assembled the hydrogel
patch with a sacrificial layer that can dissolve in media after a controlled time. This hydrogel patch self-folded into a compact tube shape and delivered via a catheter to a targeted area followed by unfolding to a patch after a particular time. Lastly, I reported an *in situ* synthesis of metal nanoparticle-hydrogel composite that can sustainably reduce the release rate of embedded metal nanoparticles. The resulting gel composite with antimicrobial property of embedded metallic nanoparticles could control bacterial cell growth in an aqueous media and also inhibit biofilm formation on a polymeric and metallic substrates coated with the gel composite. Overall, this study was conducted for enhancing the efficacy of molecular compounds used for various agricultural products, food additives, sensor devices, and clinical treatments.
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CHAPTER 1

HYDROGELS FOR MOLECULAR RELEASE: CHALLENGE AND OUTLOOK

In this chapter, first, I summarize with a fundamental overview of prior studies and approaches taken in hydrogels to enhance controlled molecular release. Second, I highlight key challenges that should be still resolved relating with the prior approaches. Finally, I propose alternative strategies to overcome such challenges and the organization for the reminder of the dissertation.

1.1 Hydrogels for drug delivery

Hydrogels are cross-linked networks of hydrophilic polymers, capable of retaining large amounts of water while remaining insoluble and maintaining their three-dimensional structure. Since their discovery and application in the biomedical field by Wichterle et al. in the early 1950s, a series of hydrogels have been developed to use them for a broad range of biomedical and pharmaceutical applications, including contact lenses, tissue engineering, diagnostics and drug delivery.[1]

Hydrogels are generally regarded as biocompatible materials because their high water content and soft nature render them similar to the natural extracellular matrix.[2] Furthermore, their porous structure along with the water content is extremely suitable to accommodate high loads of water-soluble compounds, like therapeutically active proteins and peptides, in a physiologically relevant setting.
Unlike other delivery systems (emulsified microparticles, etc.), where preparation conditions are sometimes detrimental to biomacromolecules (i.e. use of organic solvents and molecular denaturation processes, like homogenization, exposure to interfaces, etc.), hydrogel preparation procedures are beneficial in preserving molecular stability, as mild conditions (aqueous environment, room temperature) are typically adopted. Macromolecules can be physically incorporated in the hydrogel matrix, and their release is governed by several mechanisms, such as diffusion, erosion or combination of two mechanisms.[3] Hydrogels allow fine-tuning of the molecular release rate by tailoring their cross-link density via changes in polymer architecture, concentration, and the molecular weights.[4], [5]

Among hydrogels, poly(ethylene glycol) (PEG)-based hydrogels were widely researched as synthetic hydrogels because of their versatility of chemistry and biocompatibility.[6], [7] PEG is a water soluble polymer composed of repeating ethylene oxide units. Structurally, PEG is polar, uncharged, hydrophilic polymer. PEG with proper molecular weights has its properties of low protein adsorption, low toxicity, and nonimmunogenicity.[8] PEG is a versatile building block for a variety of biomaterials since its physical and chemical properties can be modified by changing molecular weight, architecture (branched, star, or comb), and functional groups.

1.2 Mechanism of PEG hydrogel formation: step-growth, chain-growth, or mixed-mode gelation

Even though diverse gelation methods (i.e., physical, ionic, or covalent cross-linking) can be used to form PEG hydrogels, covalently cross-linking gives relatively stable hydrogel structures with tunable properties like permeability, diffusivity, elastic modulus and swelling ratio. The
synthesis of covalently cross-linked PEG gels is generally processed by three categories: chain-growth, step-growth, or mixed-mode chain and step growth.[9]

The chain-growth polymerization is typically implemented with functional PEG molecules, such as poly(ethylene glycol) diacrylate (PEGDA). Reactive radicals, generated from thermal energy, redox reactions, or the initiator photo-cleavage, initiate polymerization. The chain growth gelation occurs when free radicals propagate through unsaturated vinyl bonds on the PEG monomers. One advantage of this gelation is that functionalities can be expanded through the copolymerization of other functional acrylated macromers. Photo-polymerization is one of the preferable methods to assemble PEGDA hydrogels because of the short gelation time. One possible drawback of chain-growth polymerization, compared to step-growth mechanism, is that it can have lower conversion of the functional groups. Therefore, when the chain-growth polymerization is used to from implantable gels in situ, remaining monomers or functional groups in the hydrogel may cause local inflammatory reaction or immune response. [10]

For the step-growth gelation, a multi-functionalized molecular system is cross-linked using bi-functionalized molecules in a stoichiometric manner by the reaction between the functionalities.[11] Unlike the chain-growth mechanism, this reaction can be performed under ambient conditions without initiators, and it makes fewer structural defects during network formation. This allows more precise control of gel properties like cross-linking density and stiffness. Hubbell and colleagues developed the step-growth approach to form degradable hydrogels via Michael-type reaction between acrylated star PEG polymer and dithiol.[11], [12] One advantage to this technique is that the degradation of these gels does not generate high
molecular weight chains, which sometimes have problems like host inflammatory. When it comes to macromolecular delivery, the molecular damage is not caused by the propagation of the free radicals as occurred in chain-growth polymerizations. However, the presence of thiol groups may reduce the native disulfide bonds of the biomacromolecules and cause denaturation of the molecules.

PEG hydrogels formed by mixed-mode gelations utilize characteristics between chain and step-growth gelations. This mixed-mode gelation overcomes the long gelation time required in most of the Michael-type reaction. Also, functional macromers like peptides can be incorporated at lower concentrations. Dissimilar to thiol-acrylate Michael addition reaction, thiol-acrylate photopolymerization is involved. The presence of photoinitiators increases the gelation rates as more thiol groups were added. Unlike chain-growth gelation, the initiator is not required in mixed-mode photopolymerizations; initiator-free gelation using mixed-mode has been studied.

1.3 Prior approaches to control molecular loading and release in PEG hydrogels

Drug molecules can be homogeneously loaded into hydrogel matrices through either post-fabrication (i.e., drugs are loaded by incubating gels in drug-dissolved solution after gel fabrication) or in situ encapsulation (i.e., drugs are encapsulated by cross-linking of polymers). While the post-fabrication method ensures drug stability, accurate control over the amount of drug loading is not allowed because of limited diffusion partitioning. In addition, formulating hydrogels with multiple drugs at controllable levels is problematic. On the other hand, in situ encapsulation method can be used to prepare hydrogels with controlled amount of drugs while gelating molecules such as radical initiators can be detrimental to the embedded molecules.
The drug release mechanisms from hydrogels depend on multiple factors, including the method of drug loading, the size of the molecule, and the property of molecules. One easy way to control the drug release rate from a hydrogel is to modify the gel permeability by changing cross-linking density of a hydrogel. However, this approach is limited because hydrogels have intrinsically fast diffusion. Increasing cross-linking density often reduce cytocompatibility because of decreased hydrophilicity. Therefore, other controlling mechanisms should be considered to have more refined controllability and availability of the molecular release from a hydrogel. In the past, diverse prior approaches have been conducted as summarized below:

Stimuli-sensitive hydrogel for drug delivery:
Many types of hydrogels that can change their swelling ratio in response to external stimuli have been developed and applied for the controlled drug release.[14] Physiological change (e.g. temperature, pH, and ionic strength) stimulates the hydrogel to switch from a collapsed to swollen state. When the hydrogels are in collapsed state, the matrix limits the release of encapsulated drugs. On the contrary, when the physiological stimuli reach to the hydrogel's open condition, the drugs are released.

Chemically-coupled PEG hydrogel:
Therapeutic drugs can be covalently coupled to PEG hydrogel matrices via pendant functional groups. The degradable linkers between the tether and the drug can be used for pre-determined liberation and release rates. Diverse mechanisms of degradable linker have been proposed including hydrolytic and enzymatic degradation.[15] One drawback of this approach is that the
covalent conjugation of drugs to the linker may destabilize molecules, especially when fragile proteins and peptides are used as cargos. Furthermore, partially degraded linker fragments may cause unwanted host immune responses.

PEG hydrogel having "affinity" to molecular cargos:

“Affinity” hydrogels have been suggested for controlling drug availability in PEG hydrogels. By modifying the gel-forming polymers with oligopeptides or proteins that can physically associate with molecules of interest, inert PEG hydrogels can exhibit particular affinity toward the therapeutic molecules. One advantage of affinity hydrogels is that no direct chemical modification on fragile therapeutics is needed. A variety of affinity mechanisms has been shown to be effective in controlling drug availability in PEG hydrogels. For example, cyclodextrin can be conjugated into PEG hydrogels for the controlled release of small molecular weight hydrophobic drugs.[16] Also, heparin, a highly sulfated glycosaminoglycan (GAG), has been widely implemented to fabricate affinity hydrogels due to its reversible relationship to a variety of growth factors. However, the potential immunogenicity, non-specific binding with various proteins, and bleeding of heparin further complicate the release consequence in vivo.

1.4 Goal of dissertation

Even though there have been extensive efforts to control the rate of molecular release using chemically coupling method or affinity hydrogel, these approach still encountered several challenges including the instability of molecule, uncontrollability of release direction, and the extensive labor of synthesis and purification. To resolve these problems, I introduce a new way to control the rate and direction of the molecular release by changing the hydrogel shape. A self-
folding, multi-walled poly(ethylene glycol)diacrylate (PEGDA) hydrogel tube can sustainably release encapsulated molecules exclusively from its internal wall. Molecular release through the shape-changing hydrogel efficiently delivers therapeutic molecules in a local area with a sustained manner. This technique minimizes reaction steps and chemicals, thus preventing the molecular destabilization. In addition, this self-folding technique is used for therapeutic patch delivery with a minimal invasion for treatment of a large wound and tissue defect area. Furthermore, by extending this concept of controlling the molecular release, I attempt to control the release rate of embedded metallic nanoparticles in hydrogel systems, so as to use the resulting hydrogel composite for control of bacterial infection and biofouling.

The reminder of this dissertation consists of four main chapters.

In Chapter 2, I demonstrate the assembly of self-folding hydrogels with their physical characterization. This tubular structure was obtained by in situ self-folding of a bi-layered PEGDA hydrogel patch constructed with gels of significantly different rigidity and expansion ratio. Elastic modulus and expansion ratio of each hydrogel layer were tuned to control the degree of self-folding. The radius of the resulting self-folded hydrogel was also estimated with bilayer curvature equations.

In Chapter 3, I demonstrate the ability of the self-folded gel to control direction and rate of the molecular release. Colorant molecules, bovine serum albumin (BSA) or vascular endothelial growth factor (VEGF) was used as molecular cargo. A finite element method (FEM) based simulation was also performed to address the geometrical effect on controlling molecular release.
Additionally, a self-folding hydrogel loaded with VEGF was used to demonstrate a self-folding capability \textit{in situ} and also promote neovascularization at the implanted tissue.

In Chapter 4, I present a minimally-invasive bio patch delivery system for organ therapy based on this self-folding technique. I assembled the double-layered hydrogel patch with a sacrificial layer that can dissolve in media after a controlled time. Using this gel system, I address an underlying mechanism by which the gel undergoes shape changes and also demonstrate a capability of the gel to be delivered through a catheter, then recovered its shape on a tissue membrane.

In Chapter 5, I demonstrate a novel approach to securely fasten metallic nanoparticles in a PEGDA hydrogel. I fabricated the gel composites \textit{via in situ} light-induced synthesis of silver or gold nanoparticles and cross-linking of gel-forming PEGDA. Using the gel composites, I will examine effects of the fabrication process on mechanical properties, nanoparticle retention, and anti-fouling/bacterial functionality. Finally, I will evaluate a possibility of using the gel composite as an anti-bacterial coating material of various polymeric and metallic substrates.
CHAPTER 2

ASSEMBLY OF SELF-FOLDING HYDROGELS AND EXAMINATION OF PHYSICAL PROPERTIES

Significant components of this chapter were published as “In Situ Self-Folding Assembly of a Multi-Walled Hydrogel Tube for Uniaxial Sustained Molecular Release” in Advanced Materials in 2013.[17]

2.1 Introduction

Bimetallic strips have been used for a century to convert temperature change into mechanical displacement in varied mechanical and electrical devices.[18] The strip consists of two joined strip layers of different metals that expand at different rates as they are heated. The different thermal expansions force the flat strip to bend one way if heated, and in the opposite direction if cooled below its initial temperature. Similar to the bimetallic strip, I hypothesized that a bi-layered hydrogel consisting of two hydrogels with different levels of water uptake self-folds in aqueous media. I examined this hypothesis using Poly (ethylene glycol) diacrylate (PEGDA) hydrogel with different molecular weight and concentration. PEGDA is one of the popular synthetic materials used to assemble a hydrogel using ultraviolet (UV) polymerization because of its unique hydrophilicity and biocompatibility. Also, the mechanical property and swelling ratio of the resulting gel are easily modulated by changing molecular weight and concentration.[19]
2.2 Results

2.2.1 Fabrication of self-folding PEGDA hydrogel

The bi-layered hydrogel patch was assembled by first preparing a thin hydrogel of PEGDA with a MW of 400 g mol\(^{-1}\), termed as PEGDA400, by exposing the pre-gel solution to UV light. Subsequently, another hydrogel layer was prepared over the PEGDA400 hydrogel by cross-linking PEGDA with a MW higher than 400 g mol\(^{-1}\). The polymer concentration of each layer was kept constant at 20%. Each hydrogel thickness was kept constant at 200 µm. No physical separation was found between two gel layers (Figure 2.1).

The subsequent immersion of the hydrogel patches in deionized water or phosphate buffer saline (PBS) triggered self-folding into a multi-walled gel tube, exclusively for the gel patches in which the top gel layer was prepared by cross-linking PEGDA with a MW larger than 1,000 g mol\(^{-1}\) (Figure 2.2a & 2.2b). According to the magnetic resonance image of the gel tube, the PEGDA400 gel layer laden with iron oxide nanoparticles was wrapped by the higher MW PEGDA gel layer.[20] Therefore, the internal wall of the tube consisted of the PEGDA400 gel, while the external surface was the larger MW PEGDA gel layer (Figure 2.2c).

2.2.2 Expansion ratio of hydrogel

The geometries of the gel tubes were dependent on differences in the MW and concentrations between PEGDA gel layers (Figure 2.3). Increasing the MW of PEGDA in the top layer resulted in an exponential decrease in the elastic modulus and a linear increase of the one-dimensional expansion ratio as defined in Eq. (2.1),
\[ S = \left( \frac{Q_f}{Q_i} \right)^{\frac{1}{3}} - 1 \] (2.1)

where \( Q_i \) and \( Q_f \) are the degrees of swelling of a hydrogel before and after incubation in aqueous media, respectively (Figure 2.4). Specifically, bi-layered gel patches with 2 cm length and 2 cm width were self-folded into tubes with 2 cm length (Figure 2.5). The inner radius of the tube decreased from 9 to 0.7 mm by increasing the MW of the upper gel layer from 1,000 to 10,000 g mol\(^{-1}\) (Figure 2.2a & 2.3b). The same result was obtained with hydrogel strips with the same length that were 1 mm wide. The two-layered gel strips were self-folded into tubes with 1 mm length, while the inner radius of the tube was decreased by increasing the difference in MW. Additionally, this decrease in the inner radius was inversely related to thickness of the tube wall (Figure 2.3b).

2.2.3 Control of inner radius of self-folding hydrogel with molecular weight

To understand the underlying the mechanism, the inverse dependency between the inner radius of the gel tube and the difference of MW was fitted to a mathematical model originally developed to estimate the curvature of a heat-induced bimetallic strip (Eq. (2.2)),

\[ r = \frac{h}{12\varepsilon} \left( \frac{E_1}{E_2} + 14 + \frac{E_2}{E_1} \right) \] (2.2)

where \( r \) is the inner radius of the gel tube, \( E_1 \) and \( E_2 \) are the elastic moduli of the bottom PEGDA400 gel layer and the top PEGDA gel layer respectively, \( h \) is the thickness of each gel layer, and \( \varepsilon \) is the difference in the expansion ratio between two gel layers (\( \triangle S \)).[21] This mathematical model therefore suggests that the larger \( \varepsilon \) leads to a decrease of \( r \), coupled with the \( E_1/E_2 \). Incorporating measured \( E \) and \( \varepsilon \) into Eq. (2.2) resulted in estimations of \( r \) values which decrease by increasing the difference in MW of PEGDA between the two layers, similar to the
experimental results. More interestingly, these calculated \( r \) values agreed with the experimental values (Figure 2.3b). This result, therefore, suggests that the inverse relationship between the \( r \) value and the difference in MW of PEGDA is due to changes in \( \varepsilon \) and \( E_1/E_2 \).

2.2.4 Control of inner radius of self-folding hydrogel with polymer concentration

The inner radius and thickness of the gel tube was further modulated with the polymer concentration of the PEGDA400 gel layer (Figure 2.6). According to measurements of elastic moduli and expansion ratios of the gels, decreasing the concentration of PEGDA from 30 to 10 % (w/w) significantly reduced the elastic moduli from 1.3 to 0.03 MPa and increased the degree of swelling from 5 to 10 (Figure 2.3c); however, there was a minimal change in the expansion ratio (Figure 2.7). Decreasing the polymer concentration of the PEGDA400 gel layer from 20 to 10 % (w/w) decreased the inner radius from 1.0 to 0.8 mm and also increased the wall thickness from 0.9 to 1.1 mm (Figure 2.3d). Again, this decrease in the inner radius of the gel was in agreement with the mathematical model noted in Eq. (2.2). These results show that the inverse relationship between the inner radius of the gel tube and polymer concentration of the gel is mainly related to changes in \( E_1/E_2 \).

The inner radii of self-folded gel tubes were further related to the differences of cross-linking density between two gel layers (\( N \)), calculated by Eq. (2.4) in Table 2.1. The inner radius of the gel tube was exponentially decreased by increasing difference of \( N \), which was tuned by altering difference of MW of PEGDAs used to prepare the bi-layered hydrogel patch (Figure 2.8a). In contrast, the inner radius of the gel tube was linearly increased with difference of \( N \), which was tailored by altering concentration of PEGDA400 pre-gel solution in the bi-layered gel patch.
consisting of PEGDA400 and PEGDA3400 gel layer (Figure 2.8b). Separately, cross-linking density of two gel layers was tailored to be similar to each other using PEGDA400 and PEGDA10000, in order to examine whether the gel still self-folds at zero-difference of \( N \) between two gel layers. The gel could still self-fold due to the difference of expansion ratio between two gel layers (Figure 2.9, Table 2.2). Taken together, we interpret that the self-folding mechanism is not simply explained by the difference of cross-linking density between two gel layers.

2.3 Discussion

These results demonstrate a multi-walled PEGDA hydrogel tube formed from the self-folding of a gel patch composed of two layers with different elastic moduli and expansion ratios. The differential stress between the two gel layers likely drove the self-folding process, as demonstrated through both experimental results and mathematical modeling. This self-folding process is distinguishable from other self-folding polymeric materials, with regards to the strategy of using differential stress.[22] Previously reported materials were largely constituted with chemically dissimilar materials which present different degradation rates and thermal sensitivities. Additionally, this study highlights that the difference of the expansion ratios between two gel layers determines the inner radius of the self-folded gel tube more predominantly than difference in the degree of swelling.
2.4 Conclusion

Synthesis of a bilayer hydrogel with different level of water uptake resulted in multi-walled hydrogels in aqueous media. The wall thickness and radius of resulted gel tubes were calculated from elastic modulus and expansion ratio of each gel layer. The mathematical modeling was very well matched with experimental data. This self-folding property can be attained by pairing a wider array of hydrogel systems which react at the interface and exhibit significantly different expansion ratios, stiffness, or both.

2.5 Materials and Methods

Synthesis of PEGDA:

Poly(ethylene glycol) diacrylate (PEGDA) was synthesized via a chemical reaction between poly(ethylene glycol) (PEG, Sigma Aldrich) and acryloyl chloride (Sigma Aldrich). First, PEG with varying molecular weights (MW ~ 1,000, 3,400 and 10,000 g/mol) was dissolved in dichloromethane at the concentration of 10 wt %. Next, acryloyl chloride and triethylamine (Fisher Chemical) were dissolved in the PEG solution and stirred overnight under dry N₂ gas. The molar ratio of PEG, acryloyl chloride, and triethylamine was kept constant at 1:4:4. After 24 hours, the product was precipitated by adding ice-cold ether. The crude product was dissolved in deionized (DI) water and dialyzed for one day to remove unreacted starting materials and the salt byproducts. Then, the product was freeze dried. The conjugation of acrylate groups onto PEG was confirmed by ¹H-NMR (300 MHz, QE300, General Electric). PEGDA with MW of 400 g/mol was used as received (Polysciences Inc.)

Fabrication of a self-folding bi-layered PEGDA hydrogel:
Pre-gel solutions were prepared by dissolving varying concentration of PEGDA (MW ~ 400, 1,000, 3,400, 10,000 g/mol) in DI water, with 0.01 wt.% Irgacure 2959 (Ciba specialty chemicals) as a photo-initiator. First, PEGDA400 solution was placed between two glass plates separated by a spacer with a 170 µm thickness. Then, the gel solution was exposed to UV light (Jelight Co. Model 20, maximum UV wavelength 254 nm, distance between sample and lamp 1 cm) for 5 minutes. Second, the pre-gel solution of PEGDA with MW of 1,000, 3,400, and 10,000 g/mol was placed on the PEGDA400 hydrogel surface using a spacer with 340 µm thickness. Again, the pre-gel solution placed on top of the PEGDA400 gel was exposed to UV light for 5 minutes. The resulting bi-layered hydrogel was cut into a patch with controlled widths and lengths for further characterizations. Separately, the single-layered hydrogel of PEGDA with controlled MW was prepared in the form of the disks with a 1-cm diameter and 1-mm thickness for measurements of elastic moduli and degrees of swelling.

Hydrogel characterization:

Stiffness of the PEGDA hydrogel was evaluated by measuring an elastic modulus of the gel disk. Following incubation of hydrogel disks in DI water at 25°C for 24 hours, the gel disks were uniaxially compressed at a rate of 1 mm/min using a mechanical testing system (Insight, MTS Systems). The elastic modulus was calculated from the slope of a stress vs. strain curve at the first 10% strain.

The degree of swelling was characterized with mass of water taken up by the gel. First, mass of the hydrated gel was measured after incubation in DI water over 24 hours. Then, the gel was lyophilized to measure the dried solid mass. Finally, the degree of swelling was calculated from a mass ratio of the hydrated gel to the dried solid, $Q_m$, using the Eq. (2.3),
\[ Q = v_2^{-1} = \rho_p \left( \frac{2m}{\rho_S} + \frac{1}{\rho_p} \right) \]  

(2.3)

where \( \rho_S \) was the density of water and \( \rho_p \) was the density of PEGDA.

The cross-linking density was calculated using the Eq. (2.4),

\[ N = \frac{kQ^{-1/3}}{RT} \]  

(2.4)

where \( k \) is the stiffness, \( R \) is the gas constant (8.314 Jmol\(^{-1}\)K\(^{-1}\)) and \( T \) is the temperature at which the modulus was measured.
2.6 Figures

Figure 2.1 Schematic depicting the procedure of self-folding hydrogel fabrication. (1) First hydrogel layer is formed between two glass plates with a 200μm thickness gap with UV irradiation. (2) Second layer is formed on top of the first layer. (3) Doubled layered hydrogel is immersed in DI water.
Figure 2.2 Fabrication of self-folding PEGDA hydrogel. (a) Optical images of self-folded hydrogel tubes. Images in (b) are lateral views of tubes with 20-mm length and images in (a-2) are cross-sectional views of tubes with 1-mm length. The numbers in each image represent the MWs of PEGDA used to prepare the top-layer in the bi-layered gel patch. Polymer concentrations of the bottom PEGDA400 gel layers and the top high MW PEGDA gel layers were kept constant at 20 % (w/w). All scale bars represent 1 cm. (c) A magnetic resonance image of the cross-sectional view of the multi-walled PEGDA gel tube, in which iron oxide nanoparticles are loaded into the PEGDA400 gel layer.
Figure 2.3 Effects of different molecular weights (MW) and concentrations of PEGDA hydrogels on the self-folding of the bi-layered PEGDA gel. (a) The elastic moduli (●) and expansion ratios (□) of the hydrogels prepared with PEGDA of different MWs. (b) The dependencies of the inner radii (●) and wall thicknesses (□) of the gel tubes on the difference in the MW of PEGDA gel layers. Data points represent average values of four different experiments per condition, and a dashed curve represents the simulated result. (c) Elastic moduli (●) and expansion ratios (□) dependency on the PEGDA400 concentration in the bi-layered gel patch. (d) Dependencies of the inner radii (●) and wall thicknesses (□) of the self-folded gel tube on PEGDA400 concentration of the gel layer. A dashed curve represents the simulated result. In (c) and (d), the bi-layered gel construct was constituted with the PEGDA400 gel and PEGDA3400 gel.
Figure 2.4 Schematic depicting definition of expansion ratio ($S$).
<table>
<thead>
<tr>
<th>Sample No</th>
<th>Length (mm)</th>
<th>Gel layer 1</th>
<th>Gel layer 2</th>
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<td>PEGDA2000</td>
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<td>PEGDA2000</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
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<td>PEGDA3400</td>
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**Figure 2.5** Self-folded hydrogel tubes with controlled lengths and diameters. (a) Top views of the gel tubes (b) cross-section views of the gel tubes.
**Figure 2.6** Effects of PEGDA400 concentration in the bi-layered PEGDA gel construct on multi-walled gel tube formation. In this study, the other gel layer in the bi-layered gel construct was prepared by cross-linking 20 % (w/v) PEGDA3400 solution. (a) Top and cross-sectional views of the multi-walled gel tubes with 2 cm length. Concentration of the PEGDA400 in the gel layer was varied from 10 (1st sample from the left) to 15 (2nd sample), and 20 % (w/v) (3rd sample). (b) Cross-sectional view of the multi-walled gel tubes with 1 mm length. Concentration of the PEGDA400 in the gel layer was varied from 10 (1st sample from the left) to 15 (2nd sample), and 20 % (w/v) (3rd sample). The gel tube was vertically placed on top of a glass. In (a) and (b), scale bars represent 2 mm.
Figure 2.7 Effects of concentration of PEGDA400 in the gel on the swelling ratio and expansion ratio.
Figure 2.8 Relationship between the inner radius of the self-folded gel tube and the difference of cross-linking density \( (N) \) between two gel layers (a) The dependency of the inner radii of the gel tubes on the difference of \( N \), for bi-layered hydrogels consisting of PEGDAs with different molecular weights (MWs). The top-layer of the bi-layered hydrogel patch was prepared by altering MW from 1,000 (Dot a-1), to 2,000 (Dot a-2), 3,400 (Dot a-3), and 10,000 g/mol (Dot a-4). The PEGDA concentration was kept constant at 20 % (w/v). The bottom layer of the bi-layered gel patch was prepared with 20 % (w/v) PEGDA 400 solution. (b) The dependency of the inner radii of the gel tubes on the difference of \( N \), for gel patches consisting of a layer of 20 % (w/v) PEGDA 3400 gel and a layer of PEGDA 400 gel of varying PEGDA400 concentrations. PEGDA 400 concentration was varied from 10 (Dot b-1) to 15 (Dot b-2), and 20 % (w/v) (Dot b-3).
Figure 2.9 Image of the gel tubes resulting from self-folding of the bi-layered gel patch consisting of PEGDA400 gel and PEGDA10000 gel. The concentration of PEGDA400 and PEGDA10000 were 10.3% and 20%, respectively, to minimize the difference of cross-linking density between two gel layers (Table 2.2).
2.7 Tables

Table 2.1 Experimentally measured stiffness, degree of swelling, mathematically calculated expansion ratio and cross-linking density of each hydrogels.

<table>
<thead>
<tr>
<th>MW (Conc.)</th>
<th>k (kPa)</th>
<th>Q</th>
<th>S</th>
<th>N (mol/m$^3$)</th>
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<tbody>
<tr>
<td>400 (10%)</td>
<td>29</td>
<td>11</td>
<td>0.08</td>
<td>5.2</td>
</tr>
<tr>
<td>400 (15%)</td>
<td>343</td>
<td>5.9</td>
<td>0.08</td>
<td>76.7</td>
</tr>
<tr>
<td>400 (20%)</td>
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<td>4</td>
<td>0.08</td>
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<td>1000 (20%)</td>
<td>540</td>
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<td>0.11</td>
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</tr>
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<td>10.1</td>
<td>0.21</td>
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<td>3400 (20%)</td>
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<td>18.7</td>
<td>0.44</td>
<td>18.7</td>
</tr>
<tr>
<td>10000 (20%)</td>
<td>40</td>
<td>38.9</td>
<td>0.80</td>
<td>8.6</td>
</tr>
</tbody>
</table>

k: Stiffness, Q: Degree of swelling, S: Swelling ratio, N:Cross-linking density

Table 2.2 Experimentally measured stiffness, degree of swelling, mathematically calculated expansion ratio and cross-linking density of PEGDA400-PEGDA10000 hydrogels formulated to present the minimal difference of cross-linking density between two gel layers.

<table>
<thead>
<tr>
<th>MW (Conc.)</th>
<th>k (kPa)</th>
<th>Q</th>
<th>S</th>
<th>N (mol/m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 (10.3%)</td>
<td>47</td>
<td>10.0</td>
<td>0.08</td>
<td>8.8</td>
</tr>
<tr>
<td>10000 (20%)</td>
<td>40</td>
<td>38.9</td>
<td>0.80</td>
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</table>
CHAPTER 3

GUIDE OF MOLECULAR RELEASE USING THE SELF-FOLDING HYDROGEL

Significant components of this chapter were published as “In Situ Self-Folding Assembly of a Multi-Walled Hydrogel Tube for Uniaxial Sustained Molecular Release” in Advanced Materials in 2013.[17]

3.1 Introduction

For the last several decades, hydrogels have been increasingly used to control the spatiotemporal distributions of various diagnostic and therapeutic bioactive molecules within tissues of interest through local and sustained molecular release.[9], [23] To attain this goal, extensive efforts have been made to control gel properties (i.e., degradation rates, interaction with bioactive molecules, etc) mainly by chemically modifying gel-forming polymers; however, these approaches often encounter several challenges including maintaining the structural integrity of the gels, the denaturation of macromolecules, and a limited transport of macromolecules into target tissue.[24], [25] To resolve these challenges, this study presents a simple, but unprecedented method to control the direction and kinetics of molecular release using a multi-walled poly(ethylene glycol) diacrylate (PEGDA) hydrogel tube formed by self-folding of a bi-layered gel patch. This self-folding property was attained by forming double-layered hydrogel patches with significantly different expansion ratios and elastic moduli. The diameter of the gel tubes could be predicted by an equation used to estimate the curvature of a bimorph beam. The resulting gel tubes exhibited a sustained release of molecules solely through its internal wall.
Multi-walled gel tubes were further used as a carrier of vascular endothelial growth factor (VEGF) to demonstrate that the unidirectional, sustained VEGF release significantly increased both the density and diameters of blood vessels around the implant site, compared to unfolded hydrogel patches. This multi-walled gel tube will greatly serve to improve the efficacy of various diagnostic and therapeutic molecules.

Prior approaches to control the release of molecules from hydrogels largely focused on chemically coupling molecules of interests to the gel-forming polymers using hydrolytically or enzymatically labile units.[26] Alternatively, efforts have been made to chemically modify gel-forming polymers with oligopeptides or proteins that can physically associate with molecules of interests.[27] These approaches have demonstrated several impressive results; however, multiple chemical modifications and purification steps often increase material costs, and also readily denature the encapsulated compounds.[25] Additionally, the degradation process typically reduces a gel’s rigidity and resistance to fracture, thus leading to an undesirable structural disintegration of the gel at an implantation site.[28] The degradation may also expedite denaturation of compounds due to pH changes and the formation of reactive species.[29] Separately, the interface formed between a gel implant and the target tissue acts as a physical barrier that limits molecular diffusion into target tissue.[30] Microfabrication techniques have been used to introduce microchannels in a gel's structure, in order to facilitate the release of molecules into tissues; however, there is still a need to develop simpler processes that are compatible with a wide array of cross-linking mechanism used to fabricate and assemble hydrogel structures.[19], [31]
We hypothesized that a gel patch consisting of two layers with significantly different stiffness and capacities to uptake water would self-fold into a multi-walled gel tube when exposed to aqueous media. The resulting gel tube would display a sustained molecular release exclusively from the internal wall of the multiwall system (Figure 3.1). This hypothesis was examined by assembling a bi-layered gel patch, in which each layer consisted of PEGDA with different molecular weights (MW) and concentrations. The material’s ability to control the direction and rate of molecular release was examined by encapsulating a small colorant or bovine serum albumin (BSA) into an inner hydrogel layer of the gel tube. Finally, a bi-layered PEGDA hydrogel encapsulating VEGF was implanted on a chicken chorioallantoic membrane (CAM) to evaluate the in situ gel tube formation and neovascularization.

3.2 Result

3.2.1 Comparison of macromolecular release between self-folding hydrogels and patches

The multi-walled PEGDA hydrogel tubes introduced in the Aim 1 study were used as a molecular releasing device by encapsulating bovine serum albumin (BSA) into the PEGDA400 gel layer during the cross-linking reaction. Interestingly, the resulting gel tubes more sustainably released BSA than the unfolded gel patches (Figure 3.2a). The BSA-encapsulating single-layered PEGDA400 hydrogel patches displayed a large initial burst release of BSA, followed by rapid release. Therefore, most of the BSA loaded in the gel diffused out within 5 days. In contrast, multi-walled gel tubes exhibited an 80 % lower BSA burst for the first 24 hours. The release rate quantified with a first-order kinetic approximation, $\beta$ in Eq. (3.1), was also 90 % lower for the multi-walled gel tube (Table 3.1),

$$\frac{C_{BSA}}{C_{BSA_0}} = e^{-\beta t}$$  \hspace{1cm} (3.1)
where \( C_{\text{BSA}} \) is the amount of BSA remaining in the hydrogel at time \( t \), and \( C_{\text{BSA},0} \) is the amount of BSA initially loaded in the hydrogel.[32] Similarly, VEGF was released from the self-folded gel tube more sustainably than the gel patch (Figure 3.3).

3.2.2 Simulation study of molecular release

To further examine whether BSA is locally released from the internal wall of the tube, I estimated the concentration distribution of BSA discharged from the unfolded PEGDA hydrogel patches and the multi-walled gel tubes, using a diffusion-based finite element method (FEM) (Figure 3.2a & 3.2b). Diffusion coefficients for the BSA release in simulations were calculated from the experimental values displayed in Figure 3.1a, using Eq. (3.2),

\[
\frac{M_t}{M_\infty} = 4 \left( \frac{D t}{\pi L^2} \right)^{1/2}
\]  

(3.2)

where \( M_t \) and \( M_\infty \) are the cumulative amounts of drug released at time \( t \) and at an infinite time, respectively; \( D \) is the diffusion coefficient of the drug within the system; and \( L \) is thickness of the hydrogel patch or tube wall.[32] The released BSA amount estimated by the FEM-based simulation agreed with the experimentally measured values.

This simulation also disclosed a continued increase in the local BSA concentration within the multi-walled gel tube, independent of the tube length. Furthermore, simulations were conducted to examine the diffusion of BSA and VEGF released from the multi-walled gel tubes into tissues for drug release applications. In the simulation, the self-folded gel tube with length of 1 mm was vertically implanted on tissue with the hydrogel ring and the strip as controls. The membrane implanted with the self-folded gel tube exhibited a more sustained increase of the amount of BSA than three other controls (Figure 3.4 & 3.5). The local increase of protein concentration was
attributed to the release of proteins from the inner layer of the gel tube. In contrast, other three control gel implants did not result in local increase of proteins in close proximity of gel implants.

3.2.3 Colorant release from self-folding hydrogels

To validate these simulation results, we incorporated a colorant, 2,2'-Bis(2,3-dihydro-3-oxoindolyliden), into the PEGDA400 gel to monitor direction of molecule release within a short time period. Very interestingly, the colorant loaded into the multi-walled hydrogel tube with 2 cm length was released exclusively through two open ends of the gel tube (Figure 3.2c). No significant circumferential diffusion of the BSA was observed. In the same manner, the gel tube with 1 mm length, which was vertically placed on a glass surface, also exhibited diffusion of the colorant through the internal wall (Figure 3.4c).

3.2.4 Angiogenesis study with a chicken chorioallantoic membrane

Finally, the gel tube encapsulating VEGF was implanted on a chicken chorioallantoic membrane (CAM) to test whether the local, sustained VEGF release stimulates vascularization at an implantation site (Figure 3.6). In this experiment, the bi-layered PEGDA gel encapsulating VEGF in the PEGDA400 gel layer was assembled into a strip with a 2 cm length and 1 mm width. Then, the bi-layered hydrogel strip was implanted on the CAM. The strip self-folded into a tube with 1.3-mm inner radius, 1-mm height, and 700-µm wall thickness within 10 minutes (Figure 3.7). Within 7 days, the VEGF-encapsulated self-folded hydrogel tube significantly stimulated blood vessel growth around the implant, as compared to other controls, including the VEGF-encapsulating hydrogel ring with 2.2-mm outer radius and 1.3-mm inner radius, VEGF-encapsulated hydrogel disk with 2.2-mm radius and the VEGF-encapsulating PEGDA400
hydrogel strip. The VEGF amount loaded in each gel implant was kept constant at 60 ng, in order to examine effects of gel geometry on vascularization at the same VEGF dosage.

According to previous studies, the VEGF dosage was large enough to stimulate vascularization in CAM, when VEGF was released from a hydrogel modified to degrade hydrolytically.[33] According to histological cross-sections of the tissues, the density of mature blood vessels positively stained by an antibody to α-smooth muscle actin was 1.5-fold larger for the VEGF-releasing multi-walled gel tubes than hydrogel rings, and 2-fold larger than other control conditions (Figure 3.8 & 3.9a). Additionally, there was a significant increase in the fraction of blood vessels with larger cross-sectional areas of 3,000 µm² or greater in the membranes implanted with the VEGF-releasing gel tubes, compared to other conditions (Figure 3.8 and 3.9b). The PEGDA400 hydrogel rings and disks laden with VEGF resulted in limited vessel growth and even hydrogel strips showed similar vessel growth to the VEGF-free hydrogel tubes.

3.3 Discussion

The highlight of this study is that the resulting multi-walled PEGDA hydrogel tube can sustainably release biomacromolecules solely through two open ends. I suggest that this feature is attained for the following reasons: (1) a decrease in the surface area, (2) the presence of the multi-walls, (3) a sealing of the external tube surface by the blank gel layer, and (4) heterogeneously loaded drugs in a bi-layer. The self-folding process reduced surface area of the gel layer by 84%. The BSA or VEGF release rate should be significantly decreased in proportional to the surface area. Additionally, the blank hydrogel layers between the PEGDA400 gel layers decrease the BSA diffusivity in the gel tube. Moreover, the blank gel layer on the
external surface likely acted as an insulator to prevent circumferential molecular release, similar to the myelin sheath which insulates neural axons.[34] This simple, but refined controllability of the direction and kinetics of molecular release at this time has not yet been reported using any self-folding polymeric constructs.

Furthermore, this study demonstrated that the uniaxial, sustained VEGF release from the internal wall of a gel tube is more advantageous to promote vascularization than the gel strip or disk. I interpret that the VEGF released within the core of the tube diffused into tissue and sustainably stimulated vascular sprouting, although its efficiency may be mitigated by the structure and rigidity of target tissue.[35] In contrast, limited vascularization in tissue implanted with the VEGF-encapsulated gel ring, disk or strip is likely due to the minimal localization of VEGF in implanted tissue. This result is in accordance with previous studies that address the role of VEGF localization in guiding capillary sprouting.[31]

3.4 Conclusion

Overall, the multi-walled hydrogel tube will be useful for the local sustained delivery of a wide array of drug molecules, and subsequently improve the quality of molecular therapies. The self-folded shape of gels may be readily controlled by altering geometry and shape of hydrogel patch using various microfabrication technologies. Also, this construct can further be advanced by loading multiple molecular compounds used for diagnostics, imaging, and treatments in separate layers. Instead of molecular compounds, the hydrogel can also be encapsulated with stem cells or islets capable or secreting multiple trophic factors, in order to release cell-secreted factors in a local and sustained manner.
3.5 Materials and Methods

Analysis of macromolecular release from the hydrogel:

Bovine serum albumin (BSA, Sigma Aldrich) was loaded into the bottom PEGDA400 gel layer in the bi-layered gel, in order to characterize release properties of the hydrogel. A bi-layered PEGDA400 hydrogel in which BSA was incorporated into only one of the layers was used as a control. BSA was mixed with the PEGDA400 solution at a concentration of 1 % (w/w). Then, the single-layered and bi-layered gels were prepared via photo cross-linking reaction, as described above. The gel patch with 20-mm length, 20-mm width, and 1-mm thickness and the self-folded gel tube with 2-mm radius and 20-mm length were stored in a small vial filled with 3 ml of phosphate buffer saline (PBS; pH 7.4, Cellgro), then incubated at 37 oC. 1 ml of PBS was collected from the each vial on a daily basis for 50 days, while keeping entire media volume constant at 3 ml using fresh PBS. The concentration of BSA released into PBS was determined by the bicinchoninic acid assay (Micro-BCA, Thermo Scientific). The Micro BCA assay was performed by pipetting three sample replicates (each 150 µl) to a 96-well plate, adding BCA reagent (150 µl) for each replicate and finally measuring the absorbance at 562 nm using a micro-plate reader (Tecan Infinite 200 PRO). The BSA amount back-calculated from the measured absorbance was normalized by the total amount of BSA initially loaded into the gel, in order to quantify the cumulative percentage of BSA released.

Separately, vascular endothelial growth factor (VEGF, R&D systems) was loaded into the bottom PEGDA400 gel layer in the bi-layered gel. A bi-layered PEGDA400 hydrogel in which VEGF was incorporated into only one of the layers was used as a control. VEGF was mixed
with the PEGDA400 solution at a concentration of 37.5 ng/mL. Then, the single-layered and bi-layered gels were prepared via photo cross-linking reaction, as described above. The self-folded gel tubes and gel patches were stored in a small vial filled with 3 ml of phosphate buffer saline (PBS; pH 7.4, Cellgro), then incubated at 37°C. 1 ml of PBS was collected from each vial on a daily basis for 7 days, while keeping entire media volume constant at 3 ml using fresh PBS. The VEGF concentrations in the media were measured using a VEGF ELISA kit (R&D systems), following the manufacturer’s protocol. The absorbance intensity of each well was measured at 450nm and corrected with the intensity at 570nm using a microplate absorbance reader (Synergy HT, Biotek). A calibration curve, prepared by measuring the absorbance values of standards with known concentrations of VEGF, was used to quantify the concentrations of VEGF in the collected cell culture media.

Numerical Analysis:

3D finite element models for hydrogel constructs were created using Comsol Multiphysics 4.1. It was assumed that the drug release kinetics of the unfolded hydrogel patch and the multi-walled gel tube was governed by the Fick’s law of diffusion. Four hydrogels used in the model include (1) the multi-walled gel tube with 1-mm inner radius and 20-mm length, (2) the PEGDA400 gel patch with 20-mm length and 20-mm width, (3) the multi-walled gel tube with 1-mm inner radius and 1-mm length, and (4) the PEGDA400 strip with 20-mm length and 1-mm width. The concentration of BSA in the unfolded gel patch and multi-walled gel tube was kept constant at 10 mg/ml. The CAM membrane was modeled as being 500 µm thick with a constant concentration of zero at the lower boundary of the model. This constant concentration acted as a sink, thereby consuming the BSA or VEGF once it reached the bottom surface of the model. The diffusion
across a boundary between two gel layers was assumed to be unobstructed. In these numerical analysis, diffusion coefficients of BSA and VEGF in media, tissue, PEG3400 hydrogel, and PEG400 hydrogel were approximated to be 100 µm²/s, 1 µm²/s, 0.05 µm²/s, and 0.015 µm²/s, respectively.[36], [37] All simulations were in meters, seconds, and grams. Each of the samples was modeled to simulate a period of 20 days.

Neovascularization study using CAM:

Fertilized chicken eggs (Hy-Line W-36) were obtained from the University of Illinois Poultry Farm (Urbana, IL). The eggs were initially incubated for 7 days, while being placed horizontally inside an incubator at 37 oC and 65 % humidity. Next, a hole with a diameter of 2 cm was made on the top of the egg shells, and the samples were placed on the CAMs. The eggs were incubated for 7 days at 37 oC, and the vascular networks in the CAMs were imaged using a S6E stereomicroscope (Leica) linked with a D-Lux E Camera (Leica). All CAM experiments were performed under sterile conditions. On the 7th day after implantation, the embryos were fixed with a 10% neutral buffered formalin solution (3.7 % formaldehyde in PBS). The center of hydrogels and CAMs of each sample was excised. The explants were embedded in paraffin, sectioned, and stained with an antibody to α-smooth muscle actin (αSMA). The density and numbers of positively stained blood vessels in tissue areas within a distance of 2 mm from the center of gel implants were quantified using image analysis software ImageJ (Figure 3.10). The blood vessel densities were determined by measuring the total blood vessel area, and normalizing it to the total cross-sectional area of the CAM. For each condition, 3 CAM samples were implanted and evaluated.
3.6 Figures

**Figure 3.1** Schematic depicting multi-walled PEGDA hydrogel tube that sustainably releases encapsulated growth factors in uniaxial direction and subsequently enhances neovascularization.
Figure 3.2 Experimental and computational analysis on rate and direction of molecular release from the unfolded gel patch and the multi-walled hydrogel tube. (a) The BSA release profile of the PEGDA400 gel patch (□) and multi-walled gel tube (●) consisting of a PEGDA400 gel layer and PEGDA3400 gel layer. Data points represent the average values of the experimentally measured BSA amount and dashed curves represent the simulated result. (b) Simulated concentrations of BSA through multi-walled gel tubes with 1-mm radii and 20-mm length (left image) and PEGDA 400 gel patches with 20-mm width and 20-mm length (right image) after incubation for 10 days. Arrows represent the direction of BSA release through the hollow core of the tube. (c) An optical image to exhibit unidirectional release of a colorant, 2,2'-Bis(2,3-dihydro-3-oxoindolyliden), through two open ends of the gel tube.
Figure 3.3 The VEGF release profile of the PEGDA400 gel patch (□) and self-folded gel tube consisting of a PEGDA400 gel layer and PEGDA3400 gel layer (○).
Figure 3.4 Numerical and experimental analysis of BSA distribution released into a tissue membrane, following the release from the gel tube. (a) Numerical analysis of BSA concentrations in local tissue membrane implanted with the self-folded gel tube, the gel ring and the gel strip. The analyzed area of membrane is within horizontal radius of 2 mm and vertical distance of 500 µm from the center of gel implant. (b) Simulated concentration distribution of BSA released into tissues implanted with the self-folded hydrogel tube with 1-mm inner radius and 1-mm length (I), the hydrogel ring with 2-mm outer radius and 1-mm inner radius (II) and the hydrogel strip with 1-mm width and 20-mm length (III) (lower image), after five days of implantation. The gel tube was vertically implanted. (c) Optical images of the colorant released into the hollow core of the gel tube. The units for the simulation scale bars are mmol m$^{-3}$. 
Figure 3.5 Numerical analysis of VEGF distribution released into a tissue membrane, following the release from the gel tube. (a) VEGF concentrations in CAM implanted with the self-folded gel tube, the gel ring, the gel disk, and the gel strip. The analyzed area is within horizontal radius of 2 mm and vertical distance of 500 μm from the gel implant. (b) Cross-sectional concentration distribution of VEGF in CAM implanted with the self-folded hydrogel tube with 1.3-mm inner radius and 1-mm length (I), the hydrogel ring with 2.2-mm outer radius and 1.3-mm inner radius (II), the hydrogel disk with 2.2-mm radius (III) and the hydrogel strip with 1-mm width and 20-mm length (lower image), after five days of implantation. The gel tube was vertically implanted. (IV). (c) Top view of concentration distributions of CAM implanted with each sample. The units for the simulation scale bars are μmol/m³.
Figure 3.6 Schematic depicting the in-vivo CAM experiment procedure.
**Figure 3.7** Images of the *in vivo* self-folding of the bi-layered PEGDA hydrogel strips with 1-mm width and 20-mm length on CAM.
Figure 3.8 Optical top-view images of vascular networks and microscopic images of histological cross-sections of CAMs that were stained with a marker for α-smooth muscle actin. Samples include the CAMs implanted with multi-walled hydrogel tube with 2.2-mm outer radius, 1.3-mm inner radius and 1-mm thickness (I), the CAM implanted with a PEGDA400 gel ring with 2.2-mm outer radius, 1.3-mm inner radius and 1-mm thickness (II), the CAM implanted with a PEGDA400 gel disk with 2.2-mm radius and 1-mm thickness (III), and the CAM implanted with a PEGDA400 gel strip with 1-mm width and 20-mm length (IV). All samples were laden with VEGF of 60 ng. Concentrations of VEGF in the hydrogel tube, ring, disk and strips were 7.5, 7.5, 5.0 and 15.0 µg ml⁻¹, respectively.
Figure 3.9 Enhanced vascularization with the VEGF-releasing multi-walled hydrogel tube. (a) Vascular densities of the CAM implanted with the VEGF-encapsulating gel tube, ring, disk and strip. (b) Analysis of vascular size mitigated by the VEGF-releasing devices.
**Figure 3.10** Schematic showing gel implants and analyzed area of CAM.
3.7 Tables

Table 3.1 The release rate ($\beta$) and the coefficient of determination ($R^2$) according to the first-order kinetic approximation.

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<th>$\beta$</th>
<th>$R^2$</th>
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<td>$1.68 \times 10^{-3}$</td>
<td>0.9076</td>
</tr>
<tr>
<td>Patch</td>
<td>$19.1 \times 10^{-3}$</td>
<td>0.8898</td>
</tr>
</tbody>
</table>
CHAPTER 4

BIO-PATCH DELIVERY SYSTEM

This chapter introduces the bio patch delivery system using self-folding technique.

4.1 Introduction

In the past several decades, a delivery of therapeutic nano or micro particles has shown great enhancement of therapeutic efficacy. However, particle delivery systems have had challenged with a treatment of a large local area such as organ surface treatment. Instead, researchers have made efforts to develop diverse bio-patches for therapeutics, diagnostics or imaging for the damaged tissue area.[38]-[42] For example, hydrogel bio-patches have been researched for therapeutic protein or growth factor delivery in a large local area. Similarly, stem cell sheets were studied to treat cardiovascular disease using its cell-based therapies.[31], [43] In addition, flexible electronic patches were developed to diagnose the organ's status by bonding the patch to the surface of organ.[44], [45] These bio-patches displayed impressive results and expanded the limit of nanoparticle treatment to a large local area on damaged organs. However, there have been no investigations about techniques to deliver the patches to target organ without invasive surgical procedures. Patients still need advanced materials that can be implanted on organs in a minimally invasive manner to meet the rapidly growing biotechnology.

Here, we present the easily implantable hydrogel patch that is able to self-fold and unfold. This self-folding and unfolding property was obtained by bonding the sacrificial layer, which is rapid biodegradable and expandable hydrogel, to the therapeutic layer. This bi-layered hydrogel patch
self-folds to a small form of a gel tube in a short time, then unfolds to a patch by rapid degradation of a sacrificial layer in a controlled time at an implant site. This system is similar to the commonly used long sheet delivery such as carpets or metal sheets, but do not need additional machinery for folding. For a demonstration of this patch delivering technique, we implanted a gel patch on an egg embryonic membrane using a catheter via a small invasive hole. I propose this will greatly serve to develop biomedical materials for diagnostics, imaging, and treatments of organs.

4.2 Results

4.2.1 Self-folding/unfolding hydrogel

In our previous research, PEGDA-PEI hydrogels consisting of poly(ethylene glycol) diacrylate (PEGDA, Mw=400 g mol⁻¹) and polyethylenimine (PEI), which were formed through Michael-type reaction between PEGDA and branched PEI, rapidly degraded over the course of several hours.[46] Their degradation times were controllable by changing concentration of PEGDA or PEI. We applied this unique hydrogel as a sacrificial layer functioning for self-folding/unfolding. On the other hand, PEGDA hydrogel, which have been conventionally used as a bio-patch for a drug and protein carrier, was used as a non-degradable therapeutic layer.[47] The bi-layered hydrogel patch composing of PEGDA-PEI hydrogel and PEGDA hydrogel was assembled by first preparing a thin therapeutic hydrogel layer of PEGDA (concentration of pre-gel solution was 20 weight %) with a MW of 400 g mol⁻¹, by exposing the pre-gel solution to UV light. The concentration and molecular weight of the therapeutic layer were kept constant throughout this study. Subsequently, the sacrificial hydrogel layer (PEGDA-PEI) was prepared by pouring mixed PEGDA and PEI solution over the therapeutic hydrogel. Each layer thickness was kept
constant at 200 µm. Chemical reaction between PEI and the remaining acryl functional group of prefabricated PEGDA hydrogel layer occurred. Thus, no physical separation occurred between the two gel layers. The subsequent immersion of the hydrogel patches in deionized (DI) water or phosphate buffer saline (PBS) triggered self-folding into multi-walled gel tubes, and after a certain time, the self-folded gel tube unfolded to perfectly remained only the flat therapeutic gel layer (Figure 4.1 & 4.2).

4.2.2 Physical kinetic property of PEGDA-PEI hydrogel
To investigate the geometrical change of the bi-layered gel over time depending on the sacrificial layer composition, the elastic moduli and swelling ratios of PEGDA-PEI hydrogels with varying concentrations were measured over time, and the expansion ratio was calculated by the equation,

\[ S = \left( \frac{Q_f}{Q_i} \right)^{\frac{1}{n}} - 1 \]  

Eq. (4.1)

where \( Q_i \) and \( Q_f \) are the swelling ratio of a hydrogel before and after incubation in aqueous media, respectively (Figure 4.3).[17] PEGDA-PEI hydrogel displayed a substantial increase of elastic modulus compared to only PEGDA hydrogel, and increasing PEGDA and PEI concentration significantly contributed to the increase of initial elastic modulus of PEGDA-PEI gels (Figure 4.3a-1 & 4.3b-1). However, the elastic modulus of gels rapidly decreased because of initial swelling process, then gradually approached zero by the degradation process (Figure 4.3a-2 & 4.3b-2). In the same manner, the expansion ratio initially increased without a decrease in solid mass, yet as the expansion ratio reached higher values of 0.6, the solid mass of the hydrogel started to decrease significantly (Figure 4.3a-3 & 4.3b-3). The time required for complete degradation decreased from 80 minutes to 8 minutes with increasing concentrations of PEI from 5% to 20% at a constant PEGDA concentration of 10 % (Figure 4.4a). On the contrary, the
complete degradation time was increased from 30 minutes to 210 minutes with increasing concentrations of PEGDA from 10% to 20% at a constant PEI concentration of 10 % (Figure 4.4b).

4.2.3 Radius change of self-folding/unfolding hydrogel over time

To understand the underlying mechanism of radius change of self-folding hydrogel over time, the inner radius of the gel tube over time depending on the different MW of PEGDA or PEI for PEGDA-PEI gel was fitted to a mathematical model originally developed to estimate the curvature of a bimetallic strip with same thickness layers (Eq. (2)),

\[ r = \frac{h}{12e} \left( \frac{E_1}{E_2} + 14 + \frac{E_2}{E_1} \right) \]

Eq. (4.2)

where \( r \) is the inner radius of the gel tube, \( E_1 \) and \( E_2 \) are the elastic moduli of the bottom therapeutic PEGDA gel layer and the top sacrificial PEGDA-PEI gel layer respectively, \( h \) is the thickness of each gel layer, and \( e \) is the difference in the expansion ratio between two gel layers (\( \Delta S \)) (Figure & Chapter 4.2.6 for detailed calculation).[21], [48] The calculated minimum radius (\( r \)) of hydrogels during self-folding/unfolding process agreed well with the experimentally measured values. However, changing the composition of the PEGDA-PEI gel did not significantly affect on the minimal radius than varying the thickness of hydrogel layers. For example, the minimum radius of gel tube was ranged from 0.7 mm to 1.3 mm by changing PEGDA concentration on fixed 10% PEI, and was ranged from 1.3 mm to 1.5 mm by changing PEI concentration on fixed 10% PEGDA. On the contrary, thickness of gel layer massively affected on the minimal radius of gel tube. The minimal radius was 0.5mm to 5mm when thickness changes from 40 µm to 800 µm. This is explained from the Eq. (2) that the radius is linearly proportional to layer thickness.
In addition, the complete opening times of bi-layered gel tubes were measured and compared with the degradation time of PEGDA-PEI gel (Figure 4.6). The slope of linear fitting was 0.85 and the correlation ($r^2$) between complete opening time of gel tubes and gel degradation time was measured as 0.99. This result suggests that the PEGDA-PEI homogenously degrades and the complete gel opening time was controllable by changing the composition of PEGDA-PEI gel.

4.2.4 Residual stress at the interface of hydrogel

However, PEGDA(20%)-PEI(10%) condition made the gel fracture (Figure 4.7). To find the cause of the fracture, the residual stress at the interface between therapeutic layer and sacrificial layer was calculated from the measured elastic modulus and expansion ratio by

$$\sigma_m = \frac{\Delta \varepsilon E_1 E_2}{E_1 + E_2} + \frac{hE_1(E_1 - E_2)}{2r(E_1 + E_2)}$$  \text{ Eq. (4.3)}

where $\sigma$ is the maximum residual stress at the interface, $E_1$ and $E_2$ are the elastic moduli of the bottom therapeutic PEGDA gel layer and the top sacrificial PEGDA-PEI gel layer respectively, $r$ is the inner radius of the gel tube, $h$ is the thickness of each gel layer, and $\varepsilon$ is the difference in the expansion ratio between two gel layers ($\Delta S$) (Figure 4.8 & Chapter 4.2.6 for detailed calculation).[21], [48] Calculated residual stress at the condition of 20% PEGDA and 10% PEI reached 100 kPa during the process (Figure 4.8b). The therapeutic layer at the condition was torn during the self-folding/unfolding process because the therapeutic PEGDA gel layer could not endure the residual stress. This was confirmed by the performing the tensile stress test of 20% PEGDA therapeutic layer, which the layer had the fracture at 80 kPa with the maximum strain of 0.16 (mm/mm) (Figure 4.8c & 4.8d).
4.2.5 Bio-patch delivery

For the demonstration of the bio-patch delivery system, a chicken chorioallantoic membrane (CAM) was used as a model surface of tissue. A therapeutic PEGDA gel layer with 40 μm thickness and 10 mm diameter was prepared, and same thickness of PEGDA (10%)-PEI (5%) sacrificial layer was topped on the therapeutic layer (Figure 4.9a). When the bilayer gel was immersed in phosphate buffer saline (PBS) solution, it self-folded into a gel tube with 1 cm length and 1.5 mm outer diameter. After 30 minutes of incubation in PBS, the folded tube was transferred via PTFE tubes (1.8 mm inner diameter and 2.1 mm outer diameter) through an eggshell. For transferring gel tubes, only 2.3 mm diameter of a hole was required and the hole size was significantly compared with the one (15 mm) for conventional patch delivery (Figure 4.9b). To see the process of unfolding of gel tube on CAM, the eggshell was opened and monitored with a video camera. The folded gel tube was completely unfolded on CAM within 30 minutes (Figure 4.10).

4.2.6 Calculation for radius and residual stress of bilayer hydrogel

Next are the equations for a bilayer hydrogel with different expansions. This calculation is based on the next assumptions; 1) the materials are elastic. 2) Width of bilayer could be considered very small. 3) Expansion is uniform. 4) There is no separation between two layers.

If two materials are separate, their lengths are different by different swelling ratio if there are no external forces. However, if they are strongly bonded on the surface, internal forces ($P_1$, $P_2$) are created and also, bending moments ($M_1$, $M_2$) are created. Because there are no external forces, all forces over any cross-section should be in balance.
Force balance: \( b \int \sigma \, dA = 0, \, P_1 = P_2 = P \) \hspace{1cm} \text{Eq. (4.4)}

Bending moment: \( M = M_1 + M_2 = P \left( \frac{h_1 + h_2}{2} \right) \) \hspace{1cm} \text{Eq. (4.5)}

where, \( b \) is width of two gel layer, \( h_1 \) and \( h_2 \) is the height of each gel layer.

Strain \( (\varepsilon) \) caused by bending is calculated following equation,

\[
\varepsilon = \frac{(R + y)\theta - R\theta}{R\theta} = -\frac{y}{R}
\]

where, \( R \) is the radius of the bended material, \( \theta \) is the degree of angle and \( y \) is the distance from the interface.

Bending moments for rectangular shaped materials are calculated following calculation.

\[
M_1 = \int \sigma_y \, dA = \int E_1 \varepsilon_y \, dA = \int \frac{E_1}{R} y^2 \, dA = \frac{E_2}{R} \int y^2 \, dA
\]

\[
\int y^2 \, dA = 4 \int_0^{\frac{h_1}{2}} y^2 \, b \, dy = 2b \left[ \frac{1}{3} y^3 \right]_0^{\frac{h_1}{2}} = \frac{h_1^3}{12}
\]

\[
M_1 = \frac{E_1}{R} \cdot \frac{h_1^3}{12} \cdot b, \quad M_2 = \frac{E_2}{R} \cdot \frac{h_2^3}{12} \cdot b \hspace{1cm} \text{Eq. (4.6)}
\]

At the interface, both materials' elongations are equal. The elongation includes the strain by axial force, and the strain by bending.

\[
\varepsilon_1 + \frac{P}{h_1 E_1 b} + \frac{h_1}{2R} = \varepsilon_2 - \frac{P}{h_2 E_2 b} - \frac{h_2}{2R}
\]

\[
\Delta \varepsilon = \varepsilon_2 - \varepsilon_1
\]

\[
\Delta \varepsilon = \frac{P}{h_1 E_1 b} + \frac{P}{h_2 E_2 b} + \frac{h_1 + h_2}{2R}
\]

\[
\frac{P}{b} \left( \frac{1}{h_1 E_1} + \frac{1}{h_2 E_2} \right) = \Delta \varepsilon - \frac{h_1 + h_2}{2R} \hspace{1cm} \text{Eq. (4.7)}
\]
Then, the curvature of bilayer is calculated by putting equation about $P$ from the calculation of Eq. (4.5 & 4.6) into Eq. (4.7).

$$ k = \frac{1}{R} = \frac{6E_1E_2(h_1+h_2)h_1h_2\Delta \varepsilon}{h_1^4E_1^2+4h_1^3h_1E_1E_2+6h_1h_2E_1E_2+4h_1^2h_2E_2^2} \quad \text{Eq. (4.8)} $$

If the thicknesses of both materials are equal ($h_1 = h_2$),

$$ R = \frac{h}{12\varepsilon} \left( \frac{E_1}{E_2} + 14 + \frac{E_2}{E_1} \right) \quad \text{Eq. (4.9)} $$

The curvature is proportional to the difference in elongation of the two materials and inversely proportional to the thickness of the bilayer.

Internal stress comprises two parts; 1) stress by axial force and 2) stress by bending moment, thus the internal stress is stated as $y$ function.

$$ |\sigma_y| = \sigma_x + \frac{E_y}{R} $$

$$ |\sigma_1|_{y|h} = \frac{P}{bh_1} - \frac{E_1h_1}{2R} $$

$$ |\sigma_1|_{y=0} = \frac{P}{bh_1} + \frac{E_1h_1}{2R} $$

$$ |\sigma_2|_{y|h} = \frac{P}{bh_2} - \frac{E_2h_2}{2R} $$

$$ |\sigma_2|_{y=0} = \frac{P}{bh_2} + \frac{E_2h_2}{2R} $$

The maximum stress during expansion is produced in the interface between two layers.

$$ |\sigma_1|_{y=0} = \frac{E_1}{h_1E_1 + h_2E_2} \left( h_2E_2\Delta \varepsilon + \frac{h_1^2E_1 - h_2^2E_2}{2R} \right) $$

$$ |\sigma_2|_{y=0} = \frac{E_2}{h_1E_1 + h_2E_2} \left( h_1E_1\Delta \varepsilon + \frac{h_2^2E_2 - h_1^2E_1}{2R} \right) $$
If the thicknesses of both materials are equal, maximum internal stress

$$\sigma_m = \frac{\Delta \varepsilon E_1 E_2}{E_1 + E_2} + \frac{hE_1(E_1 - E_2)}{2R(E_1 + E_2)}$$

4.3 Discussion

In chapter 2, we could understand the radius of self-folding hydrogel is determined by the elastic modulus and expansion ratio of each gel layer. Beyond the work, this study conducted the physical change of self-folding hydrogels over time with the kinetics of hydrogel degradation. PEGDA-PEI hydrogel degrades in a controlled manner by varying concentrations and this degradation determines the complete opening time and minimal radius of self-folding/unfolding hydrogels. Experimentally measured data was analyzed with relevant mathematical models. The mathematical model suggests that the inner radius of the gel decreases dominantly following the start of sacrificial layer's expansion because of increasing $\varepsilon$. However, after a certain time, decreased elastic modulus of sacrificial layer leads the increase of inner radius. This result, therefore, suggests that the relationship between the $r$ value and PEGDA-PEI degradation over time is due to kinetic changes of dominancy in $\varepsilon$ and $E_1/E_2$. Also, the slope between degradation time and complete opening was not 1, instead 0.85. This is explained that the stiffness of sacrificial layer becomes too week ($E < 20$ kPa) to endure the internal residual stress, thus fracture occurs in the sacrificial layer. Finally the gel tubes open up earlier than full degradation.

This hydrogel system for bio-patch delivery is highlighted in the regard that the safety of therapeutic layer should be guaranteed for delivering the patch. The stability of therapeutic layer was examined by monitoring with video and characterized by calculating the residual stress between two gel layers during the self-folding/unfolding process. First, because PEGDA-PEI
sacrificial layer is outer layer when it was self-folded, the therapeutic layer is protected inside during the process. Second, PEGDA-PEI degradation takes place all over the gel homogeneously, thus fairly homogeneous stress likely to be affected on entire gel during the process. This may not be achieved by other methods such as mechanical bending. Lastly, this system makes the hydrogels fold automatically without any human interaction involved. These advantages would be essential for delivering micro-sized fragile materials.

This is the first conceptual bio-patch delivery system to the best of our knowledge. This delivering technique significantly reduced required size of invasion. The self-folding/unfolding hydrogels reduced its original surface area up to 70% and also reduced the required invasion area as 2.3% to the conventional invasion area. The minimally invasive surgery is much safer way than open surgery and prevents the diverse complications and infections. Also it allows the patients to recover much faster. By combining with robotic-assisted surgery, this bio-patch delivery system will greatly serve for developing more patient-friendly surgery.

4.4 Conclusion

Overall, self-folding/unfolding hydrogel was developed for bio-patch delivery with PEGDA-PEI fast-degradable hydrogel. Because of the misfit of expansion between the therapeutic layer and sacrificial layer, the bi-layered hydrogel self-folded in a form of multi-walled gel tubes in media and unfolded with the degradation of sacrificial layer. By tuning the degradation time of sacrificial layer, self-folding and unfolding time and rate were controlled. Bio-patch delivery using this technique was demonstrated with 2.3% of invasive area than the conventional method. This bio-patch delivery system will enable clinicians to use the modern medical techniques.
4.5 Experiments

PEGDA and PEGDA-PEI hydrogels with various concentrations were synthesized and their elastic moduli and degrees of swelling were evaluated following our previous method. 20% (w/w) PEGDA (M.W. 400 g/mol) solution in DI water including 0.1% Irgacure 2959 and was first polymerized by UV irradiation with the wavelength of 253 nm for 5 minutes between glass plates with different size of gaps. Then, a PEGDA-PEI hydrogel layer was prepared on the first layer by pouring the mixture of diverse concentration of PEGDA and branched PEI (M.W. 1,000). Finally, the bi-layered gel was immersed in DI water or PBS to trigger self-folding into a hydrogel tube. For making thin film less than 200 µm thickness, a capillary force between glasses was used. Briefly, the solution was dropped on a 2 cm by 2 cm cover glass, and then a slide glass with 7.5 cm 2.5 cm was touched on top of the solution so that the solution can fill the area between the cover glass and the slide glass. A combined glass by a capillary force was hanged on supporters. The volume of solution was calculated by multiplying the needed thickness to 400 mm². The hydrogels immersed in water was recorded by a video camera (Leica D-Lux) and the radius of hydrogel was analyzed from still cuts of videos. The measured values were compared with mathematically calculated radius.
4.6 Figures

(1). Therapeutic layer
(PEGDA hydrogel or PDMS)

(2). Remained acrylate groups on surface

(3). Formation sacrificial layer on top of the therapeutic layer (PEGDA-PEI)

(4). Self-folding into a tubular hydrogel in water by expansion

(5). Unfolding in the implantation site by degradation

Figure 4.1 A Schematic image depicting the process of self-folding/unfolding hydrogel formation.
Figure 4.2 Images of self-folding/unfolding process of a bi-layered hydrogel immersed in DI water. First layer was 20% PEGDA400 gel and second layer was PEGDA(10%)-PEI(5%) hydrogel.
Figure 4.3 Degradation properties of PEGDA-PEI hydrogel with varying concentration of PEGDA and PEI. (a-1) Initial elastic modulus right after synthesis with varying PEI concentration with fixed 10% PEGDA concentration. (a-2) Elastic modulus and (a-3) expansion ratio over time with varying PEI concentration with fixed 10% PEGDA concentration. (b-1) Initial elastic modulus right after synthesis with varying PEGDA concentration with fixed 10% PEI concentration. (b-2) elastic modulus and (b-3) expansion ratio over time with varying PEGDA concentration with fixed 10% PEI concentration.
Figure 4.4 unfolding time controls by varying concentration of PEGDA or PEI. (a) The required time for self-folding/unfolding gel to be completely unfolded to the flat patch in DI water by varying concentration of PEI with fixed 10% PEGDA. (b) The required time for self-folding/unfolding gel to be completely unfolded to the flat patch in DI water by varying concentration of PEGDA with fixed 10% PEI.
Figure 4.5 Kinetics of Self-folding/unfolding process. (a-1) inner radius changes over time while self-folding/unfolding with varying concentration of PEI and fixed 10% PEGDA. (a-2) Minimal inner radius during the process of folding/unfolding from the hydrogels with varying concentration of PEI and fixed 10% PEGDA. Line is the calculated value and dots are experimentally measured data. (b-1) inner radius changes over time while self-folding/unfolding with varying concentration of PEGDA and fixed 10% PEI. (b-2) Minimal inner radius during the process of folding/unfolding from the hydrogels with varying concentration of PEGDA and fixed 10% PEI. Line is the calculated value and dots are experimentally measured data.
Figure 4.6 Correlation between complete unfolding time and full degradation time of PEGDA-PEI gel layer.

Figure 4.7 Image of cracked hydrogel tube during the self-folding/unfolding process. Sacrificial layer was PEGDA(20%)-PEI(10%). Scale bar represents 1 mm.
Figure 4.8 Residual stress calculations at the interface of two gel layers. (a) Residual stress changes of therapeutic PEGDA gel layer over time at the interface between two gel layers from the hydrogels with varying PEI concentration with fixed 10% PEG or (b) varying PEGDA concentration with fixed 10% PEI. (c) A tensile test of therapeutic PEGDA gel layer. Red dash line represents the fracture stress. (d) Maximum residual stress of therapeutic PEGDA gel layer during the self-folding/unfolding process bi-layered with the PEGDA-PEI hydrogels having different PEGDA concentration with fixed 10% PEI. The red line represents the fracture stress of therapeutic PEGDA gel layer.
Figure 4.9 (a) Images of bio-patch delivery process on CAM through an eggshell. PEGDA(10%)-PEI(5%) was used for sacrificial layer. (b) Comparison of invasion area between (b-1) self-folding/unfolding hydrogel technique and (b-2) conventional open delivery.
Figure 4.10 Images of unfolding process of self-folding/unfolding hydrogel on CAM.
CHAPTER 5

IN SITU IMMOBILIZATION OF METALLIC NANOPARTICLES IN A RIGID HYDROGEL FOR ANTI-FOULING/BACTERIAL COATING

In this chapter, I demonstrate a novel approach to immobilize metallic nanoparticles in a PEGDA hydrogel. Simultaneous light-induced synthesis of silver nanoparticles and cross-linking of gel-forming PEGDA solution resulted in a rigid hydrogel sustainably usable for anti-fouling/bacterial coating material.

5.1 Introduction

Bacterial contamination characterized by uncontrolled microbial growth in water and also on various industrial, household, and biomedical products negatively impacts on human health and welfare.[49]-[53] Metallic nanoparticles such as silver nanoparticles (AgNP) have been extensively studied to control the bacterial contamination.[54]-[56] Metallic ions dissolved from nanoparticles with a high surface area to mass ratio can damage bacterial cell membrane and also limit cell growth.[57], [58] These nanoparticles are often immobilized in a hydrogel, such that the resulting hydrogel composite can sustainably release metallic ions and subsequently control bacterial cell adhesion and growth over an extended time period.[59], [60] These nanoparticles are typically loaded into a pre-formed hydrogel via infiltration.[61] Alternatively, metal ionic precursors are infiltrated into a pre-made hydrogel, followed by activating reduction to form
metallic nanoparticles in the hydrogel.[62], [63] Conversely, gel-forming polymers mixed with pre-formed nanoparticles are cross-linked to assemble a gel composite.[64]

The resulting composites demonstrated impressively enhanced anti-bacterial properties compared with free nanoparticles suspended in aqueous media or blank hydrogels. However, nanoparticles often rapidly diffuse out of the gel matrix, partly because of the absence of association between nanoparticles and a hydrogel matrix.[65] Such uncontrolled particle loss from the gel necessitates loading of a large amount of nanoparticles to attain desired performance, thus unintended raising concerns on health and environmental impacts of nanoparticles as well as material costs. Additionally, the increased loading of pre-formed nanoparticles reduces cross-linking density of the gel, thus leading to significant decreases of mechanical stiffness and strength of the composite.

To resolve these challenges, we hypothesized that simultaneous photo-activated synthesis with metallic nanoparticle-binding polymers would create a hydrogel composite with enhanced nanoparticle retention and minimal matrix softening. The resulting AgNP-laden gel composite will present improved anti-bacterial/fouling properties even with a smaller particle loading than that conventionally used. We examined this hypothesis by exposing a mixture of poly (ethylene glycol) diacrylate (PEGDA), vinylpyrrolidone (VP), and metallic salt solutions including silver nitrate to ultraviolet (UV) lights (Figure 5.1). The poly(vinylpyrrolidone) (PVP) is known to bind with silver nanoparticles via the coordination complex and van der walls interaction, while PEGDA allows us to control mechanical stiffness of the gel composite with its concentration.[66]-[68] A photo initiator activated by UV lights is capable of simultaneously
activating radical reduction of metal ions and cross-linking reaction of the PEGDA and VP. The nanoparticles formed in a gel were examined microscopically and spectroscopically while monitoring mechanical properties of the gel in parallel. Finally, we evaluated the extent to which the gel composite controls bacterial growth in broth media suspended with the gel composites and also biofilm formation on the gel. We further used the gel to coat polymeric/metallic substrates, in order to demonstrate usefulness of the gel as an anti-bacterial/fouling coating material.

5.2 Result

5.2.1 In situ fabrication of metallic nanoparticle-laden hydrogel composites

Exposing the PEGDA solution mixed with silver nitrates to UV light resulted in hydrogels loaded with silver nanoparticles in situ, as demonstrated with color changes of the hydrogels and transmission electron microscopy images of the nanoparticles (Figure 5.2a & 5.2b). The average diameter of AgNP created in the hydrogel was approximately 8.5 ± 1.1 nm (Figure 5.2b & 5.2c). Similarly, photo-induced reduction of gold (III) chloride dissolved in the PEGDA solution also resulted in the gold nanoparticle (AuNP)-hydrogel composite. The diameter of AuNP formed in the gels was around 11.8 ± 2.0 nm. Both AgNP and AuNP formed in the gel presented a face-centered cubic crystal structure with a lattice constant of 4.1 Å, as characterized with selective area diffraction patterns (Figure 5.3).

The resulting AgNP and AuNP-hydrogel composite displayed the absorption in a visible area at the wavelength of 420nm and 530nm, respectively (Figure 5.4). Such surface plasmon resonance absorption was similar to the nanoparticles formed via conventional reduction of metal ions in
aqueous media.[69], [70] These nanoparticles remained stable in a gel over a month without any aggregation or precipitation. In contrast, nanoparticles prepared in the aqueous media aggregated within a few days, despite the presence of citrate-based capping molecules on the nanoparticle surface (Figure 5.5).

The number of nanoparticles formed in the PEGDA hydrogel was further controlled with the irradiation time. The height of absorption peak at 420 nm was measured to estimate the concentration number of nanoparticles formed in a hydrogel following the principal of Beer-Lambert law,

\[ A = -\log_{10} \frac{I}{I_0} = \varepsilon I c \]

Eq. (5.1)

where A represents absorbance; \( I_0 \) and \( I \) are the light intensity before and after passage through the gel, \( \varepsilon \) is molar absorptivity, \( l \) is thickness of the gel through which the light passes, and \( c \) is concentration of nanoparticles.[71] At a given silver nitrate concentration, the absorption peak at 420 nm was increased with the irradiation time, specifically between 5 and 12 minutes (Figure 5.6a-1). In parallel, the number of cross-links for the PEGDA hydrogel was also increased with irradiation time, as demonstrated with the increase of UV absorbance below 400 nm wavelength. The peak represents \( n - \pi^* \) to \( \pi - \pi^* \) transition bands corresponding to a cross-linking reaction. (Figure 5.6b).[72] Similarly, the peak height at 350 nm was substantially increased between 5 and 12 minutes.

The increase of the peak height was fit to a logistic curve, which follows an autocatalytic reaction model,

\[ Y = \frac{Y_M Y_0}{(Y_M Y_0) e^{-kt} + Y_0} \]

Eq. (5.2)

where \( Y \) is peak intensity at a given time \( t \), \( Y_M \) and \( Y_0 \) are maximum and minimum peak intensity values, \( k \) is the growth rate of nanoparticles or cross-links (Figure 5.6a-2 & 5.6b-2).[73] According to the calculation, \( k \) for the AgNP was 0.66 min\(^{-1}\), while \( k \) for the cross-links in the gel
was 0.69 min\(^{-1}\). These results therefore confirm that nanoparticles and gel were formed at the same rate.

The number of nanoparticles formed in the gel was also controlled with concentration of metal ionic precursors. Both the yellow color intensity and the height of UV absorption peak of the AgNP-hydrogel composites increased with the concentration of silver nitrate (Figure 5.7).

Further incorporation of VP into the PEGDA hydrogel slowed down both the reduction rate of nanoparticles and polymerization because the UV light at the wavelength of 253 nm, which was used for \textit{in situ} gelation and particle polymerization, was likely absorbed by the PEGDA-PVP hydrogel more than the PEGDA hydrogel (Figure 5.8). However, after completion of the reaction in 30 minutes, minimal difference of the number of AgNP was found between the PEGDA hydrogel and the PEGDA-PVP hydrogel.

5.2.2 \textit{In situ} nanoparticle assembly minimally changes stiffness of the AgNP-hydrogel composite

This \textit{in situ} assembly of the AgNP-hydrogel composite minimally changed mechanical property of the hydrogel. Elastic moduli of both AgNP-PEGDA hydrogel composite and pure PEGDA hydrogel were approximately 900 kPa (Figure 5.9a). Interestingly, the elastic modulus of the gel composite was independent of silver precursor concentration in the pre-gelled mixture. The swelling ratio of the gel was also independent of the silver nitrate concentration. The same finding was made with the \textit{in situ} AuNP-PEGDA hydrogel assembly (Figure 5.9b). These independencies of elastic modulus and swelling ratio on the silver precursor concentration were also observed with the PEGDA-PVP hydrogel (Figure 5.9c).
In contrast, the control condition, which was prepared by mixing pre-made AgNP with pre-gelled solution followed by activation of cross-linking reaction of PEGDA, displayed the linear decrease of an elastic modulus with silver precursor concentration (Figure 5.9a). Ultimately, the PEGDA solution mixed with pre-made AgNP at concentrations higher than 2 mM failed to form the gel. Again, the significant decrease of the gel stiffness was also found when the gel composite was prepared with the pre-made AuNP (Figure 5.9b). The inverse dependency of the elastic modulus on silver nitrate concentration was also observed with the PEGDA-PVP hydrogel composite.

Overall, according to the number of cross-links in the gel calculated using an elastic network model (see Eq. (5.5) in the experimental section), in situ gel composite assembly minimally influenced the cross-linked structure of the gel, while the pre-made nanoparticles substantially reduced the number of cross-links between polymers (Figure 5.9d). The same results were also found with the PEGDA-PVP hydrogel system (Figure 5.9e).

5.2.3 Analysis of nanoparticle retention in the hydrogels

The gel composite prepared by the in situ assembly displayed enhanced retention of nanoparticles as compared with the two-step process in which pre-made nanoparticles were added into the PEGDA solution followed by activation of cross-linking reaction. Interestingly, during incubation of the gel composites in DI water over 5 days, the concentration of AgNP in the PEGDA gel, calculated with the UV absorbance decreased by 5 % (Figure 5.10a). During the same period, the hydrogel composite prepared by the two-step process displayed the release of more than half of the AgNP.
Separately, the gel’s capability to retain nanoparticles was evaluated by measuring contents of AgNP and silver ions in incubation media using the inductively coupled plasma-mass spectroscopy (ICP-MS). The gel composite prepared via *in situ* assembly exhibited much smaller releases of AgNP and ions than that prepared by the two-step process (Figure 5.10b). The release rates ($\beta$) of AgNP and ions were further quantified using a first-order kinetic approximation [Eq. (5.3)]

\[
\frac{M_t}{M_\infty} = 1 - e^{-\beta t}
\]

Eq. (5.3)

where, $M_t$ and $M_\infty$ are the cumulative amounts of particles or ions released at time $t$ and at an infinite time, respectively. The gel composite prepared via *in situ* assembly displayed 15 and 3-fold lower $\beta$ values for AgNP and Ag$^+$ ions, respectively, than that prepared via the two-step process.

More interestingly, addition of VP into the AgNP-PEGDA gel composite system further reduced both released amounts and rates of AgNP and Ag$^+$ ions. Specifically, few AgNP were released from the PEGDA-PVP hydrogel prepared by the *in situ* assembly (Figure 5.10b-1). The $\beta$ value of Ag$^+$ ions was also 10-fold smaller than that for the gel composite prepared by the two-step process (Figure 5.10b-2).

### 5.2.4 Antibacterial activities of the AgNP-hydrogel composite

The function of the AgNP-hydrogel composite to control bacterial growth was evaluated by measuring bacterial growth rates in media immersed with the composite. The AgNP-gel composites were immersed in the lysogeny broth (LB) media suspended with gram negative bacteria *Escherichia coli* (*E.* coli) at a density of $1.5 \times 10^6$ (CFU)/ml. Without the gel composite,
the cell number analyzed with the optical density at 600 nm increased to $10^9$ (CFU)/ml within 8 hours (Figure 5.11a-1). The cell growth rate ($G_R$) was quantified by fitting the curve of optical density over time to a modified Gompertz growth model (Eq. (5.4)),

$$Z = Z_0 + Z_M \exp \left\{ -\exp \left[ \frac{e^{G_R}}{Z_M} \times (L_g - t) + 1 \right] \right\}$$  \hspace{1cm} \text{Eq. (5.4)}

where $Z$ is the viable cell count (log CFU/ml), $Z_0$ is the initial log number of cells, $Z_M$ is the difference between the initial and final cell numbers, $L_g$ is the lag time before the cell growth, and $t$ is the sampling time.[74]

The AgNP-PEGDA gel composite immersed into the cell media significantly influenced both $L_g$ and $G_R$. Increasing the initial silver concentration in the gel composite from 0 to 10 mM exponentially increased $L_g$ (Figure 5.11a-2). $G_R$ was also decreased with increasing silver concentration (Figure 5.11a-3). In particular, the PEGDA gel composite loaded 10 mM silver, inhibited an increase of the cell number over 5 days.

More interestingly, the AgNP-PEGDA-PVP gel composite further limited the cell growth even at the silver concentration of 0.2 mM (Figure 5.11b-2). The $L_g$ was approximately 16 hours, which was comparable to that attained with the 2 mM AgNP-PEGDA hydrogel composite. The $L_g$ was also about 3-fold longer than the 0.2 mM AgNP-PEGDA gel composite. The $G_R$ was also significantly decreased with addition of VP into the gel composite (Figure 5.11b-3). In contrast, the pure PEGDA-PVP hydrogel made minimal influences on both $L_g$ and $G_R$.

Additionally, gel composites incubated in DI water over ten days were challenged with fully saturated *E. coli* ($10^9$ CFU/ml), in order to assess the long-term anti-bacterial activities of the gel composites. Only AgNP-PEGDA-PVP hydrogel composites prepared by the *in situ* assembly
lysed the bacterial cells by 80% and ultimately made the opaque bacterial cell suspension clear (Figure 5.12a).

The assembly method and composition of the gel composite also orchestrated to control biofilm formation on the gel surface (Figure 5.13). As characterized with the tdTomato fluorescence-expressing E. coli, the pure PEGDA hydrogel allowed active bacterial cell adhesion and growth on its surface during incubation at 37°C over 7 days (Figure 5.13-I). The AgNP-PEGDA gel composite prepared by the two-step process significantly decreased the number of fluorescent E. coli on the gel; however, a certain number of bacterial cells adhered to the gel surface and underwent growth (Figure 5.13-II). In contrast, both the AgNP-PEGDA gel composite and AgNP-PEGDA-PVP gel composite prepared by in situ assembly inhibited cell adhesion and growth (Figure 5.13-III & IV).

5.2.5 Evaluation of the AgNP-hydrogel composite as an anti-bacterial coating material

The resulting AgNP-PEGDA-PVP hydrogel composite was used as an anti-bacterial/fouling coating material for various substrates including nylon and aluminum foil (Figure 5.14a). A thin, AgNP-hydrogel composite layer could be prepared by first coating substrates with poly(glycidyl methacrylate) (PGMA) and then spraying pre-gelled mixture followed by UV irradiation (Figure 5.14a).[75] Interestingly, the coated nylon and aluminum substrates could minimize adhesion of bacterial cells, when they were challenged with the fully saturated (10⁹ CFU/ml) tdTomato fluorescence-expressing E. coli at 37°C over 10 days (Figure 5.14b & 5.14c). In contrast, uncoated substrates displayed strong red fluorescence from metabolically active bacterial cells. According to electron microscopic images of E. coli collected from the incubation media, cells
incubated with uncoated substrates kept their cell membrane intact (Figure 5.14d-1). In contrast, cells incubated with the coated substrates were mostly punctuated and lysed (Figure 5.14d-2).

5.3 Discussion

Taken together, this study successfully demonstrated that photo-activated in situ AgNP and PEGDA-PVP gel assembly is advantageous to fabricating a sustainable anti-bacterial/fouling hydrogel composite. We suggest that the gel softening caused by loading of pre-fabricated nanoparticles in the pre-gel solution is attributed to the limited cross-linking reaction between gel-forming polymers particularly on the nanoparticle surface. Accordingly, the resulting gel would present reduced number of interconnected polymeric networks responsible for elastic response. As the number of defects is increased with nanoparticle concentration, the gel becomes softer proportionally to the number of particles loaded into the gel. In addition, uncontrolled aggregation between nanoparticles in the pre-gelled mixture would signify the gel softening. In contrast, the in situ nanoparticle synthesis and gelation minimized the nanoparticle-induced interferential effects on cross-linking between polymers likely due to balanced nanoparticle growth within space between cross-links. This interpretation is supported by formation of fairly monosized nanoparticles and also minimal aggregation within the gel matrix. Therefore, this in situ assembly method enabled us to decouple the inverse dependency between mechanical properties of the gel composite and nanoparticle concentration, and further allow us to broadening the range of nanoparticle loading. In addition, this in situ one-step assembly allows us to avoid using reducing agents for nanoparticle synthesis. There is a possibility that the reducing agents remain in a gel matrix and negatively impact on environment and human
health.[76] To the best of our knowledge, such nanoparticle-induced matrix softening was neither addressed nor resolved to date.

Furthermore, this study demonstrated that the resulting AgNP-PEGDA-PVP hydrogel composite could be used as an anti-bacterial material to inhibit bacterial growth in a media and also biofilm formation on material surfaces even with a smaller dose of AgNP than that used in past studies. Such result was achieved by the enhanced binding between AgNP and the gel matrix, as confirmed with minimal release of nanoparticles and ions from the gel matrix. We suggest that the improved nanoparticle and ion retention in the gel is caused by coordination between of tertiary amides of PVP with silver ions.[66] Therefore, the AgNP and ions retained in the gel composite could effectively damage membrane of bacterial cells exposed to the gel surface. The underlying mechanism should be further systematically examined in future studies, because this is the first-time to demonstrate the importance of sustained presence of silver ions in a matrix for enhanced anti-bacterial control. In the past, certain studies reported that a material designed to rapidly release silver ions is more favorable to controlling bacterial cell growth in aqueous media.[77]

5.4 Conclusion

In conclusion, this study demonstrated that an advanced method to assemble the AgNP-PEGDA-PVP hydrogel composite that is structurally durable and also controls bacterial adhesion and contamination with reduced amount of AgNP. The independency of the composite stiffness on the nanoparticle concentration, attained by the in situ assembly, was attributed to the balanced growth of nanoparticles and cross-linking reaction of polymers. The superior anti-bacterial
activity of the gel composite was attributed to the sustained presence of AgNP and Ag\textsuperscript{+} ions in the gel matrix, likely due to the coordination between AgNPs and PVP of the gel matrix. As such, polymeric and metallic substrates coated by the gel composite successfully inhibit biofilm formation on the surfaces. Overall, the AgNP-PEGDA-PVP gel composite developed in this study would also greatly serve to take the quality of anti-bacterial control to a next level, with reduced concerns on the use of AgNP. Additionally, the results of this study would be broadly useful to improving a ratio of performance to particle loading for a wide array of metallic nanoparticle-laden polymer composite systems.

5.5 Method

*In situ* assembly of AgNP or AuNP-hydrogel composites:

The pre-gelled mixture was prepared by dissolving PEGDA (MW 1,000 g/mol, Polysciences Inc.), vinylpyrrolidone (VP, Sigma-Aldrich), Irgacure 2959 (0.1%, Ciba-Giegy), and silver nitrate or gold (III) chloride. Concentrations of PEGDA and VP were kept constant at 20 and 10 %, respectively. Concentrations of silver or gold salts were varied from 0.2 to 10 mM. The pre-gelled mixture was placed between two glass plates separated by a spacer with 1 mm thickness. Then, the mixture was exposed to UV light (Jelight Co. Model 20, maximum UV wavelength 254 nm) for 10 minutes. The distance between the pre-gelled mixture and the UV lamp was kept constant at 1 cm. The resulting AgNP or AuNP-hydrogel composites were cut into 5 mm-diameter disks for further analysis.

Transmission Electron Microscope (TEM) analysis of AgNP or AuNP-hydrogel composites:
The hydrogel samples were fixed in a Karnovsky's Fixative in phosphate buffered 2% glutaraldehyde and 2.5 % paraformaldehyde and then washed in phosphate buffer saline solution. The samples were dehydrated in a series of increasing concentrations of ethanol. Acetonitrile was used as the transition fluid between Ethanol and the Epoxy. Infiltration series was done with an epoxy mixture using the epon substitute Lx112. The resulting blocks were polymerized at 75 °C overnight, trimmed and ultrathin sectioned with diamond knives. Sections were examined with a TEM (Jeol 2100), at 200 kV acceleration voltage.

Analysis of mechanical properties of the hydrogel composites:

The elastic modulus of the gel composite was measured by uniaxially compressing the samples at a rate of 1 mm per minute using a mechanical testing system (MTS Insight). The slope of the stress versus strain curve was used to calculate the elastic modulus from the first 10% of strain. The number of cross-links (N) in the gel was calculated from the degree of swelling and elastic modulus using an elastic network model,

\[ N = \frac{SQ^{-1/3}}{RT} \]  

(5)

where \( S \) is the shear modulus calculated from the slope of the \( \sigma \) versus \((\lambda - \lambda^{-2})\) curve assuming that the hydrogel follows an affined network model (\( \sigma \): stress, \( \lambda \): strain), \( Q \) is the degree of swelling, \( R \) is the gas constant (8.314 Jmol\(^{-1}\)K\(^{-1}\)) and \( T \) is the temperature at which the \( S \) and \( Q \) were measured.[78]

Inductively coupled plasma-mass spectroscopy (ICP-MS) analysis:

To measure the total amount of nanoparticles formed in hydrogels, 0.5 cm\(^3\) of composites were prepared and rinsed three times for 12 hours. Then, AgNP-hydrogel composites were incubated...
in 2% ammonium persulfate solution to bleach out metallic nanoparticles in the gel. The silver concentration of the collected solution was measured using the ICP-MS (PerkinElmer - SCIEX ELAN DRCe). Separately, to measure release rates of AgNPs and Ag⁺ ions, 0.5 ml of hydrogel composites were prepared, rinsed three times for 12 hours, and incubated in centrifuge tubes with 6 ml of DI water at room temperature. 1 ml of supernatant was collected every 24 hours and total amount of silver was analyzed by ICP-MS. Another 4 ml of supernatant was collected every 24 hours. The nanoparticles were filtered through Amicon ultra centrifugal filter units (Cut-off MW ~ 10,000 g/mol) by centrifugation. Then, silver ion content in the media was analyzed by the ICP-MS. The amount of released nanoparticles was calculated by subtracting the measured silver ion mass from the total silver mass.

Analysis of anti-bacterial properties of AgNP-hydrogel composites:

Fresh E. coli (K12 TB1) was inoculated on an agar gel plate and incubated at 37 ºC for a day, so the cells form colonies. One colony was transferred to 5 ml of LB media in a tube using a sterile pipette tip. The tube was vigorously shaken at 37 ºC for 10 hours. The bacterial suspension was diluted to 1.5 x 10⁶ (CFU)/ml at which the optical density at wavelength of 600nm (OD₆₀₀) became 0.01. The 20 µl of hydrogel composites were added into 96 well plates, followed by loading 150 µl of dilute bacterial suspension. The absorbance at wavelength of 600nm was measured for 24 hours using a microplate reader (Tecan Infinite 200 PRO), while shaking the plate by 2 mm amplitude. The absorbance was used to quantify the lag time and the rate of bacterial growth. The assay was performed with four replicates for each sample.

Preparation of E. coli expressing tdTomato Fluorescence
Competent *E. coli* were obtained using a mix & go *E. coli* transformation kit (Zymo Reseach). 0.5ml of fresh *E. coli* cultured overnight in LB was inoculated to 50 ml Super Optimal Broth (SOB) medium in a 500 ml culture flask. The culture flask was shaken vigorously at 37 °C until the OD₆₀₀nm became 0.6. The culture flask was then transferred to an ice bath. After 10 minutes, the cells were centrifuged at 3,000 rpm for 10 minutes at 0 - 4°C to make a pellet. The pelleted cells were suspended in 5 ml ice-cold 1X Wash Buffer and re-pelleted, again. Then, cells were re-suspended in 5 ml ice-cold 1X Competent Buffer. 50 µl of the competent cell suspensions were aliquot to sterile centrifuge tubes and stored in a -70 °C freezer for later uses. For transformation of cells, 1 µl of ptdTomato fluorescence plasmid DNA (Clontech Laboratories Inc.) was added to 50 µl of competent cells. The mixture was placed onto an agar-gel plate containing 0.1% Ampicillin (Gold Biotechnology Inc.) and incubated at 37 °C. After 24 hours, red-colored colonies were grown on the agar-gel plate.

Analysis of anti-bacterial property of AgNP-hydrogel composites:

*E. coli* engineered to express tdTomato fluorescence was cultured in LB media supplemented with 0.1% Ampicillin for 24 hours before the experiment. AgNP-hydrogel composites with the size of 2 cm length x 2 cm width x 0.2 mm thickness were placed on the agar gel plate containing 0.1% Ampicillin. 100 µl of engineered *E. coli* was placed on surface of the hydrogel composites. The plate was incubated at 37 °C for 7 days. After the incubation, the gel composites were detached from the agar gel. Then, cells attached to the gel composites were imaged using a laser scanning confocal microscope (Carl Zeiss LSM 700). The experiment was performed with two duplicates for each condition.
Analysis of the long-term anti-bacterial property of the AgNP-hydrogel composites:

20 mm$^3$ of AgNP-hydrogel composites were incubated in DI water at room temperature for 10 days. DI water was replaced every day. The hydrogels were transferred to a 96 well plate. 150 µl of fully saturated *E. coli* suspension with $10^9$ CFU/ml was added into each well. The plate was incubated at 37 ºC without shaking, so cells readily sediment on gel surfaces. After 24 hours, cells on the gel composites were imaged with an optical microscope. In parallel, OD$_{600}$ of the each well was measured with a plate reader. The experiment was performed with 4 duplicates for each condition.

Coating of nylon and aluminum substrates with AgNP-hydrogel composites:

First, poly(glycidyl methacrylate) (PGMA) was synthesized by radical polymerization of glycidyl methacrylate (GMA, 97%, Sigma-aldrich). The mixture of 30% GMA and 1% of azobisisobutyronitrile (AIBN, 98%, Sigma-aldrich) in methyl ethyl ketone (MEK) was stirred at 60 ºC for 6 hrs. The synthesized PGMA was purified by repeated precipitation in diethyl ether, followed by drying in vacuum. Nylons and aluminum foils treated by plasma cleaner (Harrick Plasma PDC-32G) for 1 min were dip-coated in 1% PGMA dissolved in MEK, and subsequently annealed at 110 ºC for 15 min. Next, the substrates were finally immersed in the acrylic acid to link PGMA with acrylic acid. Finally, pre-gelled mixture of PEGDA and VP was placed on surface of acrylic group-functionalized substrates followed by irradiation with UV light for 10 min. Then, the resulting substrates coated by the AgNP-hydrogel composites were washed with DI water to remove excess unreacted chemicals.
Analysis of anti-bacterial properties of substrates coated by AgNP-hydrogel composites:

Both coated and uncoated substrates were placed in a 6-well plate. 3 ml of fully saturated (10^9 CFU/ml) *E. coli* expressing tdTomato fluorescence was introduced into the well. The samples were incubated at 37 °C for 10 days. After the incubation, samples were gently rinsed with DI water 3 times and fluorescence from cells attached to the surface was visualized using a stereo fluorescence microscope (Nikon SMZ800). The fluorescence of substrates was measured using a plate reader (Tecan Infinite 200 PRO), in parallel. The substrates were excited at wavelength of 540 nm, and the subsequent emission yield at 581 nm was measured. The experiment was performed with 4 duplicates for each condition.

Morphological analysis of *E. coli* with a scanning electron microscope (SEM):

The *E. coli* accumulated on substrates were collected by a sterile pipette tip and fixed in 4% formaldehyde solution. The cells were washed with DI water and centrifuged at 1,000 rpm for 5 min. Then, the cells were dehydrated by repeatedly incubating them in ethanol-water mixture with increasing ethanol concentrations (i.e., 35%, 50%, 75%, and 100%). Between each step, the samples were centrifuged at 3,000 RPM for five minutes. The dehydrated cells were finally placed on a carbon tape and coated with platinum for charge dissipation. The morphologies of *E. coli* were observed using a scanning electron microscopy (Hitach 4800).
Figure 5.1 (a) A chemical reaction scheme of the photo-activated reduction of metallic salts and cross-linking of PEGDA-PVP. (b) A scheme depicting microstructural changes of the pre-gelled mixture to a nanoparticle-laden hydrogel composite.
**Figure 5.2** *In situ* assembly of AgNP or AuNP-hydrogel composites (a) Optical images of AgNP and AuNP-hydrogel composites. 0.2mM of silver nitrates or gold (III) chloride was mixed with the pre-gel solution to form the hydrogel composites. (b) TEM images of the hydrogel composites laden with AgNP (left image) and AuNP (right image) formed via *in situ* assembly. (c) Size distribution of AgNP and AuNP formed in the hydrogel composites. Size was analyzed using TEM images.
Figure 5.3 Diffraction patterns of (a) AgNP and (b) AuNP formed in the PEGDA hydrogel and lattice constant calculations.
Figure 5.4 UV/Vis absorption spectra of AgNP and AuNP formed in the hydrogel matrix.
Figure 5.5 Optical images of the suspension of citrate-capped AgNP. The images were captured right after synthesis (a-1) and after 24 hours (b-1). TEM images of sodium citrate-capped AgNP captured right after synthesis (a-2) and after 24 hours (b-2).
Figure 5.6 Analysis of in situ AgNP formation kinetics in the hydrogel (a-1) UV/Vis absorption spectra of AgNP formed by exposure of the aqueous mixture of 0.2 mM silver nitrate and PEGDA to UV light. (a-2) Increase of the absorbance intensity of AgNP at wavelength of 420 nm with the irradiation time. (b-1) UV/Vis spectra of the PEGDA hydrogel acquired at different irradiation time points. (b-2) Increase of the normalized peak intensity at wavelength of 350 nm over time. In (a-2) and (b-2), data points were normalized by feature rescaling method in the range of [0%, 100%] and fit to the sigmoidal fitting.
Figure 5.7 Control of concentration of nanoparticles formed in the gel by varying concentration of metal ionic precursors. (a) Optical images of the AgNP-PEGDA hydrogel composite prepared with varied concentrations of AgNO₃. (b) UV/Vis absorption spectra of the AgNP-PEGDA hydrogel composite prepared with varied concentrations of silver nitrate. The spectra were acquired after UV irradiation of pre-gelled mixture over 10 minutes.
Figure 5.8 (a) UV/Vis spectra of the PEGDA hydrogel and the PEGDA-VP hydrogel. (b) Comparison of the absorbance intensity at wavelength of 250 nm between the PEGDA and the PEGDA-PVP hydrogels.
Figure 5.9 Analysis of physical properties and cross-linking density of the AgNP-PEGDA hydrogel composites (a) Elastic moduli of the PEGDA hydrogel composites laden with controlled amount of AgNP prepared either by "in situ" assembly, or "two-step" assembly. The "in situ" assembly was conducted by exposing the aqueous mixture of metallic salts and gel-forming polymer. The "two-step" assembly was conducted by incorporating pre-fabricated AgNP into a PEGDA solution followed by cross-linking reaction. (b) Elastic moduli of the PEGDA hydrogel composite laden with AuNP prepared either by "in situ" assembly, or "two-step" assembly. (c) Elastic moduli of the AgNP-PEGDA-PVP hydrogel composite prepared either by "in situ" assembly, or "two-step" assembly. (d) Cross-linking density of the AgNP-PEGDA hydrogel composites and (e) AgNP-PEGDA-PVP hydrogel composites prepared either by "in situ" assembly, or "two-step" assembly. Data points and error bars represent average values and standard deviation of 3 different samples.
Figure 5.10 Analysis of capabilities of the PEGDA and PEGDA-PVP hydrogels to retain metallic nanoparticles and ions in the hydrogel (a) Changes of the AgNP concentrations in a hydrogel prepared either by "in situ" assembly, or "two-step" assembly. The nanoparticle concentrations were calculated from the UV/Vis absorbance at the wavelength of 420 nm. (b) Accumulated ICP quantification of AgNP (b-1) and Ag+ ions (b-2) released from the hydrogel composites. In (b-1) and (b-2), "In situ+VP" indicates that the AgNP-PEGDA-PVP hydrogel composite prepared by the in situ assembly.
Figure 5.11 Antimicrobial activities of the AgNP-hydrogel composites against E. coli strains in media. (a) Changes of the optical density at wavelength of 600 nm (OD<sub>600</sub>) over time, when E. coli growth in the broth media was challenged by the AgNP-hydrogel composites prepared with different concentrations of silver nitrate. Initial E. coli concentration was 1.5 x 10<sup>6</sup> (CFU)/mL. The lag time (L<sub>t</sub>) (a-2) and the growth rate (G<sub>r</sub>) (a-3) of cells, challenged with gel composites prepared with different silver nitrate concentrations. (b) Changes of OD<sub>600</sub> over time when E. coli growth in the broth media was challenged by the AgNP-hydrogel composites with different compositions. The lag time (b-1) and the growth rate (b-2) of cells challenged with gel composites with different compositions. “None” represents the cell suspension not exposed to any gel composites, “PEGDA” does that incubated with the pure PEGDA gel, “+VP” does that incubated with the pure PEGDA-PVP hydrogel, “+Ag” does that incubated with the AgNP-PEGDA gel composite, and “+Ag+VP” does that incubated with the AgNP-PEGDA-PVP gel. All gels were prepared via the in situ assembly.
Figure 5.12 Evaluation of the long-term anti-bacterial activity of the hydrogel composites. Hydrogel composites were incubated for 10 days in DI water before adding them into the cell suspension. (a) Optic images of E. coli cell suspension challenged by the gel composites over 24 hours. “Two step” and “In situ” represent the AgNP-PEGDA hydrogel composites prepared by the two-step and in situ assembly with 0.2 mM silver nitrate, respectively. (b) Absorbance intensity of cell suspension measured at wavelength of 600 nm.
Figure 5.13 Fluorescence images of the hydrogel surface exposed to the tdTomato fluorescence-expressing E. coli. “PEGDA” represents pure PEGDA hydrogel. “Two step” and “In situ” represent the AgNP-PEGDA hydrogel composites prepared by the two-step and in situ assembly with 0.2 mM silver nitrate, respectively. “In situ + VP” represent the AgNP-PEGDA-PVP hydrogel composites prepared by in situ photo-activated cross-linking reaction. The images were captured after incubation over 7 days.
Figure 5.14 Mitigation of biofilm formation on nylon and aluminum substrates using the AgNP-PEGDA-PVP hydrogel composite as a coating material. (a) A scheme depicting sequential surface activation of target substrates (step 1) followed by immobilization of the AgNP-PEGDA-PVP hydrogel composite. (b) Optic (left) and fluorescence (right) images of tdTomato fluorescence from the uncoated and coated substrates. Images in (b-1) represent the nylon substrate and those in (b-2) do the aluminum substrate. (c) Quantification of the fluorescence yield from the nylon and aluminum substrates either uncoated or coated by the AgNP-PEGDA-PVP hydrogel composites. (d) SEM images of bacteria collected from the nylon surface either uncoated or coated by the gel composites.
CHAPTER 6

CONCLUSION

In this study I summarized prior approaches taken to enhance a PEGDA hydrogel’s ability to control molecular release and underlined key challenges (i.e., molecular damage and an uncontrollability of release direction) that should be still solved. As alternative methods, I developed the advanced in situ formations of a PEGDA hydrogel as for biomacromolecular delivery and metallic nanoparticle carrier. The significance of this study lies in (1) incorporating the idea and science of bimetallic strips into fabricating the self-folding multi-walled hydrogel, (2) controlling the release rate and direction of macromolecular drugs with the self-folding hydrogel for revascularization therapies, (3) introducing therapeutic patch delivery system that can be applied to diverse bio-patches like flexible electronic devices and cell sheets using the self-folding technique with degradable hydrogel, and (4) controlling release of metal nanoparticles in a hydrogel for anti-bacterial/fouling applications via in situ assembly of metal nanoparticles-hydrogel composites. Overall, this study should greatly serve to enhance the efficacy of multiple, molecular compounds used for diverse agricultural products, food additives, sensor devices, and clinical treatments.
REFERENCES


