

Synthesis and Characterizations of Stimuli-Responsive
Polymeric Materials for Biomedical Applications

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Degree

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Abstract

Stimuli-responsive polymeric materials have been now widely researched toward the biomedical applications including therapeutic delivery, bio-sensor surface modification, and tissue-engineering, etc., considering their desirable biocompatibility, tunable properties, and sensitivity toward physiological stimuli. Beyond the mono-responsive materials, polymers with responsiveness simultaneously toward multiple stimuli are paid great attention to because the control of responsive behaviors could be achieved at a more accurately and delicately level in a complex local environment. However, many challenges still exist such as maintaining integrity of the structure, shaping the morphology at micro- and macro-scale, and regulating a controllable and predictable transition behavior.

The objectives of this dissertation are to develop and optimize multi-responsive polymeric materials toward the physiological stimuli including temperature, pH, and oxidative stress, in the forms of nano-/micro-scaled particles, nano-scaled multilayers, and macro-porous scaffolds.

Specifically, micro/nano-scale polyetheramine containing hydrogel particles with pH-, thermo-, and oxidation-responsiveness were developed via thermally induced phase separation for therapeutic delivery (Chapter 2). The physical properties, including size, surface charge density, swelling behaviors are tunable via varying the preparation parameters such as monomer ratio, reaction time and temperature, monomer concentration, and monomer composition. Cytocompatibility and drug loading capability were investigated, and proved these particles to be of great potential for biomedical applications.

For construction of dual-responsive multilayers, pH sensitivity was incorporated into a thermo-responsive polymer (poly(*N*-vinyl-2-caprolactam)) (PVCL) via bearing a *tert*-butoxycarbonylmethyl group at the 3-position of VCL through the approach of nucleophilic substitution (Chapter 3). The biocompatibility exhibited in acute cytotoxicity assay and the successful layer-by-layer self-assembly with a cationic polymer suggested a possible application for bio-sensor surface modification.

Macro-porous hydrogels with pH-, thermo-, and oxidation-responsiveness were successfully fabricated via thermally-induced phase separation of prepolymers toward the application as scaffolds for neuronal regeneration (Chapter 4). The hydrogels exhibited micro-sized interconnected cavity for diffusion of nutrition and metabolites, hydrophobic domains for therapeutic delivery, great biocompatibility in direct contact assay, and cationic functional groups for survival of neuronal cells as well as neurite outgrowth, indicating a probable use for neuronal regeneration.

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Chapter 1 Introduction

Polymeric materials show great biocompatibility and bioactivity for biomedical applications, including implants and medical devices for replacing and/or restoring tissues, organs or functions of body, due to its low mechanical strength, low mass density, and flexibility in chemistry. It is significant that polymers are able to be functionalized with many chemical groups and incorporate sensitivities to physiological stimuli, including pH, temperature, oxidation, etc.

1.1 Physiological stimuli

1.1.1 Temperature

Temperature responsive polymers are one of the most studied class of stimuli-responsive materials. The main application is based on the fact that temperature *in vivo* may change upon fever or local diseases, therefore the difference or shifting could be a signal to induce the treatment such as local drug release. For example, compared with healthy tissues, the temperature of tumor extracellular environment is commonly 1~2 °C higher [1]. Such a narrow temperature variance is enough to induce phase separation of a polymer solution with a lower critical solution temperature (LCST) around physiological temperature. The LCST is a temperature, below which the polymer solution is homogeneous, above which the polymer solution becomes phase separated [2]. During the course of phase separation, the affinity of polymer to solvent decreases, leading to the conformation adopting of polymers.

For example, the most well-known thermo-sensitive polymer, poly(*N*-isopropy

-lacrylamide) (PNIPAAm) exhibits phase separation behavior in aqueous solution when the temperature is higher than its LCST (Scheme 1).

For the dilute polymer aqueous solutions, the phase separation behavior can be predicted using free energy of mixing ΔG_M . The ΔG_M can be obtained from the enthalpy of mixing ΔH_M and entropy of mixing ΔS_M using the equation below:

$$\Delta G_M = \Delta H_M - T \Delta S_M$$

The enthalpy of mixing ΔH_M can be estimated following the equation below:

$$\Delta H_M = V_M \left[\left(\frac{\Delta E_1}{V_1} \right)^{\frac{1}{2}} - \left(\frac{\Delta E_2}{V_2} \right)^{\frac{1}{2}} \right]^2 \nu_1 \nu_2$$

Where V_M represents the total volume of the solution, ΔE is the energy of vaporization to a gas at zero pressure while V is the molar volume of the components. The ν represents the volume fraction of component in the mixture. The subscript 1 represents the polymer while the subscript 2 represents the water molecules.

Then, the affinity of polymer with solvent can be defined as a unitless quantity χ_1 which is related to the ΔH_M :

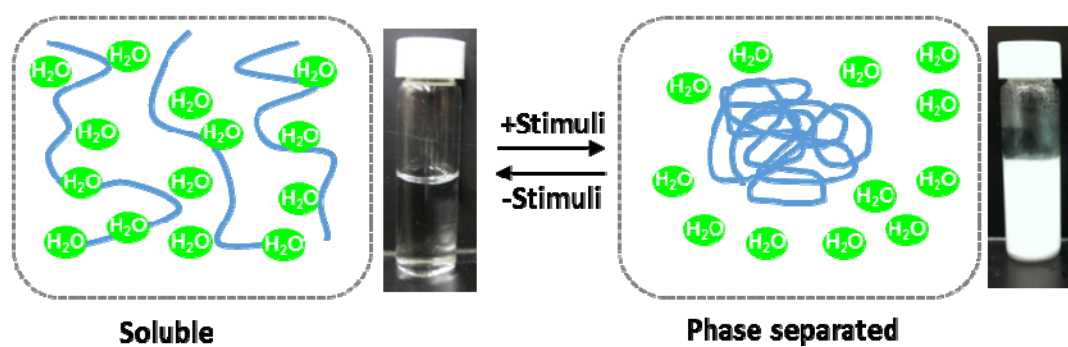
$$\chi_1 = \frac{\Delta H_M}{k T N_1 \nu_2}$$

where the N_1 is the numbers of molecules of the polymer.

Therefore the free energy of mixing in statistical thermodynamic terms:

$$\Delta G_M = k T (N_1 \ln \nu_1 + N_2 \ln \nu_2 + \chi_1 N_1 \nu_2)$$

The chemical potential $\Delta \mu_1$ of polymer can be obtained using the equation below:



Scheme 1 Inverse solution behavior of responsive polymers. Left hand side shows hydrated polymers in homogenous solution. Right hand side shows folded chains of polymers when phase separation occurs.

$$\Delta\mu_1 = \left(\frac{\partial \Delta G_M}{\partial N_1} \right)_{N_2}$$

For a phase separation, the two phases, including the polymer dominant phase a and solvent dominant phase b , with different compositions (v_1^a and v_2^a) and (v_1^b and v_2^b) are in thermodynamic equilibrium. Therefore the chemical potentials of each component should be equal respectively:

$$\begin{aligned}\Delta\mu_1^a &= \Delta\mu_1^b \\ \Delta\mu_2^a &= \Delta\mu_2^b\end{aligned}$$

In addition, for the boundary between the homogeneous phase and the phase separation, the ΔG_M follows the equation below:

$$\frac{\partial^2 \Delta G_M}{\partial v_1^2} = 0$$

The critical point on this boundary should follow the equation below:

$$\frac{\partial^3 \Delta G_M}{\partial v_1^3} = 0$$

Therefore the critical value χ_c is obtained below:

$$\chi_c = \frac{1}{2} \left(\left(\frac{v_2}{v_1 N_2} \right)^{\frac{1}{2}} + 1 \right)^2$$

Then the phase separation behavior can be explained using the factor χ_1 . When the $\chi_1 < \chi_c$ the mixture is always miscible in one phase, while the $\chi_1 > \chi_c$ would lead to

phase separation of the solution. The temperature and concentration would influence the transition behavior. As a typical phase diagram of polymer solutions, the Figure 1 demonstrates the lower critical solution temperature (LCST) and upper critical solution temperature (UCST).

PNIPAAm, as shown in Figure 2a, was first reported by Guillet et al. [3] in 1968, has attracted great attention because its LCST is around 32 °C, which is close to the typical body temperature, 37 °C. In recent decades, the PNIPAAm was well studied in the research for drug delivery, by copolymerizing or block grafting with various monomers or polymer chains, to tune the LCST and incorporate other stimuli-responsiveness. In 2005, it was found that the PNIPAAm may not be the best choice for *in vivo* drug delivery because of its moderate cytotoxicity [4]. The hydrolysis of the amide groups of PNIPAAm could produce small toxic amide compounds.

The poly(*N*-vinyl caprolactam) (PVCL) is later studied as another temperature responsive polymer for biomedical application (Figure 2b). The PVCL was considered as an alternative of PNIPAAm, because PVCL would not hydrolyze into amide moieties. The homopolymer of PVCL shows phase separation behavior in the range of 32-34 °C.

The copolymers of poly(ethylene oxide) (PEO) (Figure 2c) and poly(propylene oxide) (PPO) (Figure 2d) also exhibit phase separation behavior. PEO units are highly hydrophilic while PPO units are hydrophobic. Therefore, the LCST of copolymers of PEO and PPO ranges from 20 °C to 85 °C depending on the copolymer compositions. The commercially available PEO-PPO copolymers are Pluronics, Poloxamers and Tetronics.

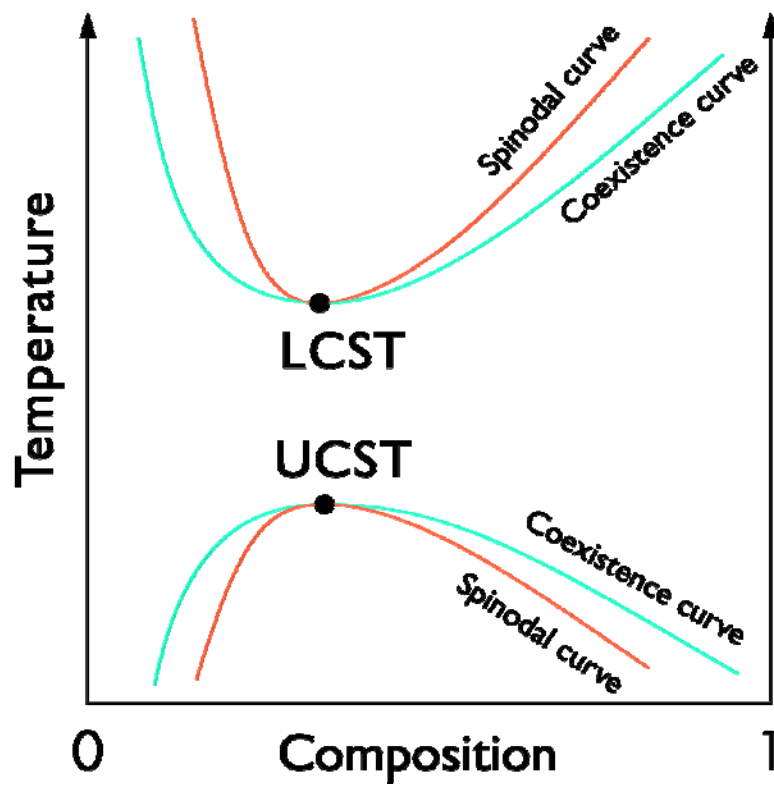


Figure 1 An example of phase diagram of polymer solutions
(https://en.wikipedia.org/wiki/Lower_critical_solution_temperature).

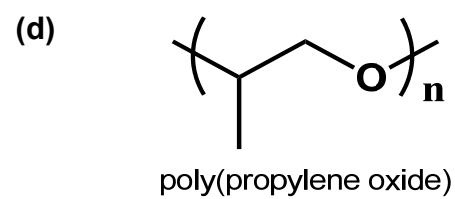
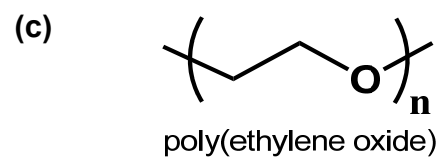
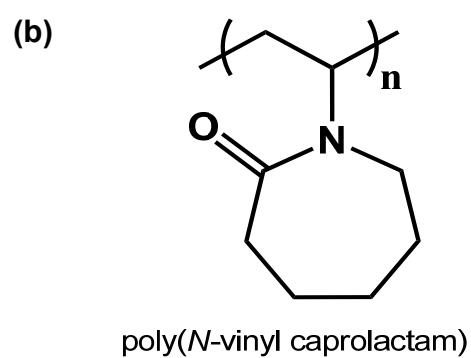
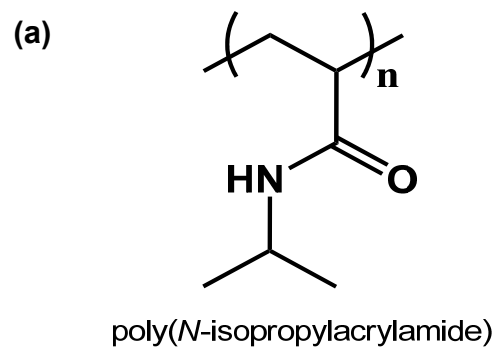


Figure 2 Chemical structures of (a) poly(*N*-isopropylacrylamide), (b) poly(*N*-vinyl caprolactam), (c) poly(ethylene oxide), (d) and poly(propylene oxide).

1.1.2 pH

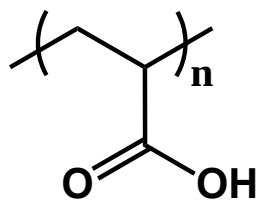
The pH-sensitivity is widely studied as a triggering factor for controlled release and drug delivery. The stimulus can be from the difference between the normal tissues and diseased tissues. For example, the average pH of cancer cells microenvironment is between 6.0 and 7.0 whereas that of healthy tissue is around 7.4. Polymers with weak acid or alkaline groups (e.g. carboxylic acid and amine groups) show pH sensitivity. These functional moieties have different threshold values, at which the switches would occur between association and dissociation with protons. The charged groups alter the solubility of the polymers, and hydrodynamic size as well.

Poly(acrylic acid) (PAA) is one of the most well-known pH-sensitive polymer with pK_a at 4.5 (Figure 3a). Above its pK_a , the carboxylic acid group deprotonates. Therefore the repulsion force between the neighboring charges keeps the polymer chain expanded. The opposite behavior was found from polybases, such as poly(2-(diethylamino)ethyl methacrylate) (PDEAEMA) (Figure 3b). The PDEAEMA solution shows phase separation when the pH is above its pK_b .

1.1.3 Oxidative stress

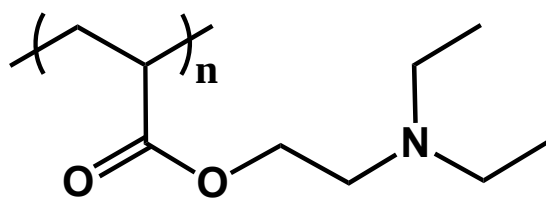
The oxidative stress is essentially a disturbance in balance between the reactive oxygen species (ROS) and the antioxidant species. The reactive oxygen species (ROS) are found at higher concentrations in cancer cells and inflammatory tissues than in healthy cells. For example, hydrogen peroxide (H_2O_2) is a common oxidative compound distributed widely, and there are slightly higher amounts near the tumor cells because of their rapid metabolism [5]. The oxidation could trigger release of drug in two approaches, including breakage and non-breakage of polymeric backbone of drug vehicles [6].

(a)



poly(acrylic acid)

(b)



poly(2-(diethylamino)ethyl methacrylate)

Figure 3 Chemical structures of (a) poly(acrylic acid) and (b) poly(2-(diethylamino)ethyl methacrylate).

For example, a non-breakage system is presented by Hubbell et al. [7] that poly(propylene sulfide) (PPS) transforms from hydrophobic chain to hydrophilic poly(propylene sulfoxide) and poly(propylene sulfone) after oxidation (Figure 4a). The hydrophilic chain would expand in the aqueous solution and then release drugs.

An example of backbone-breaking oxidation-responsive materials is phenylboronic ester-containing polymer. The phenylboronic acid (ester) could be oxidized into phenol and boric acid by H_2O_2 (Figure 4b). Therefore the phenylboronic ester-containing backbone would degrade and then release the drugs.

1.2 Multi-responsive polymers

Instead of mono-responsive systems, more and more attention has been attracted to prepare materials with response to multi-stimuli, to mimic activities of living tissues or organs and follow the pattern of natural metabolism. It is still a challenge in synthesis of a multi-responsive polymer with a controllable and predictable transition behavior [8].

The most common dual responsive polymers have been reported focused on the stimuli of temperature and pH. The NIPAAm was copolymerized with negatively charged carboxylic acid containing monomers [9-14] and positively charged amino group containing monomers [15-21]. Besides, the combination of pH and redox potential was developed via incorporating disulfide structure with amino esters for micelles targeting doxorubicin delivery [22].

Multi-responsive systems responding to more than two stimuli are of great advantages because there are extra tools for fine tuning the behaviors in a complex local environment [8]. Triply-responsiveness toward pH, temperature, and glucose concentration was achieved by Jiang et al. in the form of surface brushes [23] and by

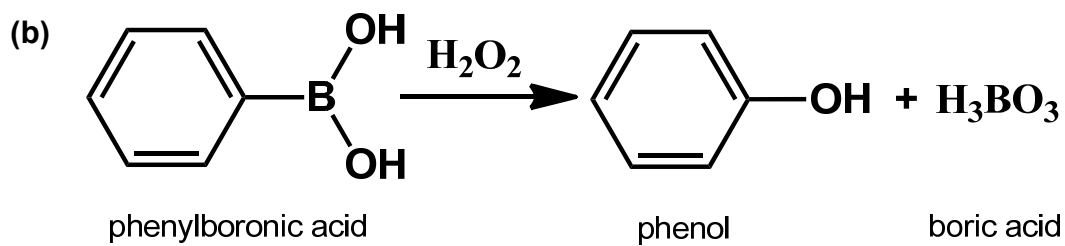
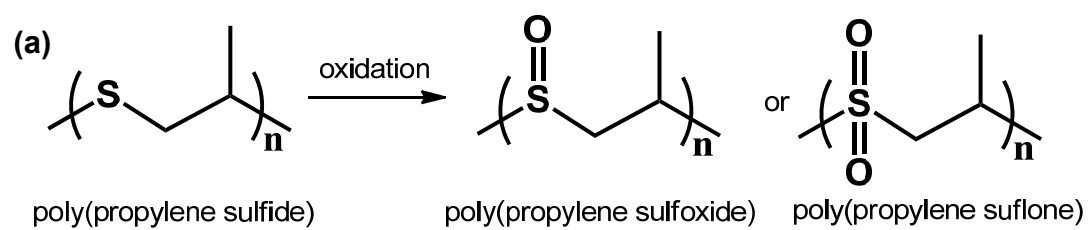


Figure 4 Oxidation of (a) poly(propylene sulfide) and (b) phenylboronic acid, and their oxidation products.

Sumerlin et al. as free polymers [24]. In addition, along with pH and temperature, the third responsiveness was reported including salt [25], redox potential [26], and enzymatic reaction [27].

1.3 Applications of responsive polymers

1.3.1 Therapeutic delivery

In conventional therapy, drugs are delivered via the routes including oral, inhalation, and injection. Through these routes, drugs will enter blood circulation system, diffuse in the body of patients, and then be consumed by the tissues or be depleted via the metabolism. So the concentration of the drugs would rise to the peak value quickly right after the treatment, and then decline gradually [28]. But for each drug there is a concentration level above which it is effective and below which it is ineffective for the therapy. In addition, some drugs are even toxic above certain concentration. Therefore, constant rate drug delivery system was developed and paid great attention to. In this system, the drug concentration will be steady for a sufficient period to strengthen the therapeutic effects.

However, though many bioactive drugs are effective to inhibit or harm the diseased tissues or cells, at the same time they are effective in a toxic fashion to the healthy tissue and cells [29]. Such side-effects are especially obvious in chemotherapy for cancer. For example, doxorubicin is commonly used to treat cancer but it can cause a series of adverse effects including hair loss, myelosuppression, nausea, vomiting, and diarrhea, etc.

In order to reduce the side-effects of therapeutic drugs, the idea of target-specific delivery was raised and widely accepted. The specific delivery would enhance the

concentration ratios of target to non-target tissue, prolong the drug residence at the target site, and therefore improve the cellular uptake by the target cells.

To load biomedical drugs, differentiate the pathological and healthy tissues, and release the payload to the target location, stimuli-responsive nano-sized vehicles are the future candidates.

1.3.2 Biosensors

Biosensors are very important for gathering *in-situ* information of a patient for better planning therapeutic treatment. In recent decades, biosensors have been greatly developed for determining the pH, and concentration of oxygen, carbon dioxide, glucose, and lactate, etc [30]. A main problem for the functioning of biosensors is the foreign body response against implanted sensor tips. At the very beginning of tip implantation, the adsorption of proteins, including albumin, fibrinogen, and fibronectin, etc., occurs to the sensor surface [31]. The fibrinogen and fibronectin contribute to formation of thrombus blood clot, and formation of provisional matrix [32]. This fibrin network containing provisional matrix provides a substrate of complex three-dimensional structure for cell adhesion and migration. The foreign body response will progress as acute and chronic inflammation, granulation tissue, and eventually fibrosis/fibrous capsulation of biosensors. The fibrous capsulation would result in a complete failure of sensing function [30]. To maintain the function of biosensor and avoid unnecessary adhesion of cells, it is critical to protect the tip surface from the adsorption of proteins.

Traditionally, the coatings to protect biosensors are realized via grafting a highly-hydrophilic layer composed of PEO structure to reduce the adhesion of proteins [33]. However, the simply hydrophilic protecting layer is far from the demands of the

biosensing system. The implantation would cause the variance of targeting parameters, malfunction of tissue, and death of local cells. So to reduce these effects, sensitive surface was studied for behavior according to the local environment. A temperature sensitive poly(*N*-vinyl-2-caprolactam) based drug delivery system [34] was reported by Cao et al. as well as a poly(*N*-vinyl pyrrolidone) based pH sensitive material for anti-inflammatory drug delivery [35].

1.3.3 Tissue engineering

Tissue engineering is defined as a combination of cells and materials via engineering process and biochemical approaches to fabricate a functioning scaffold for implantation, to improve biological functions of tissues or even to prepare a substitute of the organs [36]. In recent years, more and more researches are focused on stimuli-responsive scaffolds instead of inert-biomaterials. Stimuli-responsive hydrogels exhibit greater capability in mimicking the extracellular matrix (ECM) environment [33].

For example, a thermoresponsive matrix was reported by Ohya et al. via grafting PNIPAAm to hyaluronic acid [37]. At physiological temperature, the polymer film serves as a noncell-adhesive matrix with an extended coil of polymer branches providing stability and space-occupying structure to the extracellular matrix of all tissues.

1.4 Dissertation structure

There is a large family of polymers, which can be employed in the forms of micelle, particle, multilayer, hydrogel and solid bulk for various application. Here in this dissertation three forms of biomedical polymeric materials, including micro/nano-sized particles, nano-scale multilayers, and macro-scale hydrogel, will be presented in terms of synthesis, characterization, and application. According to the applications of each device,

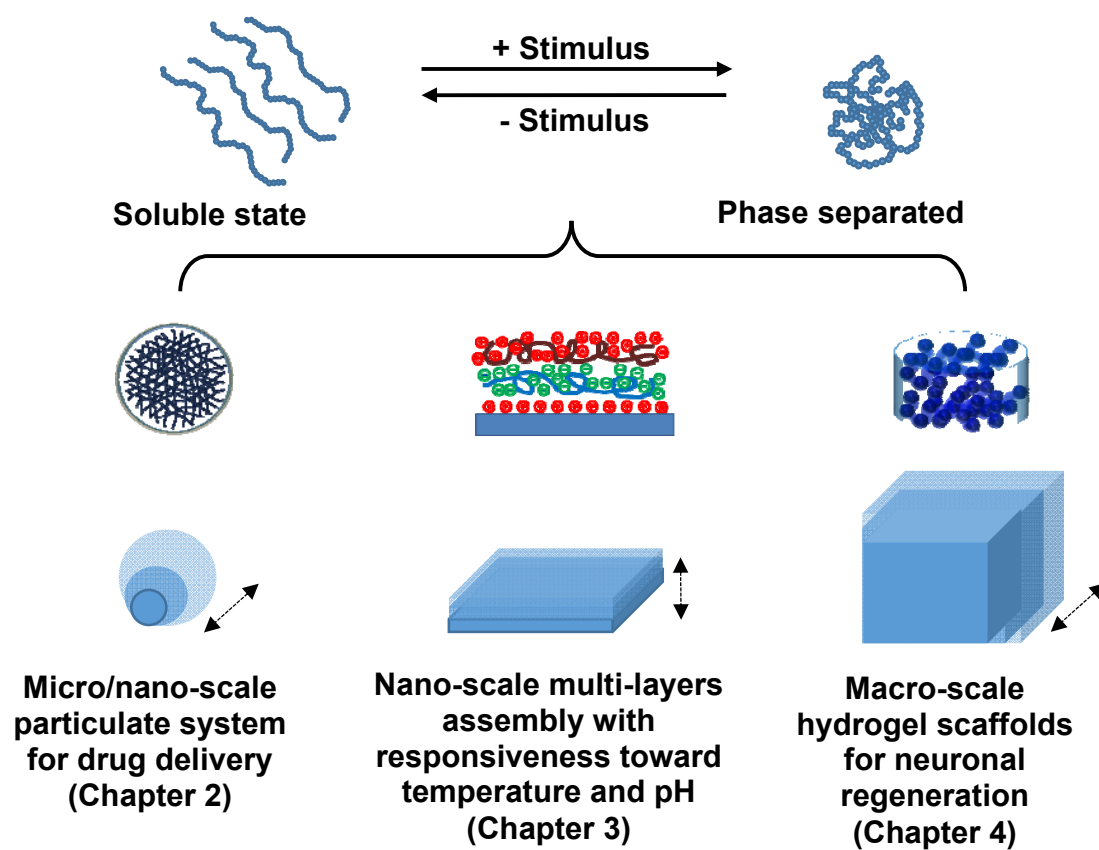
multi-responsiveness of each material was designed, incorporated, and realized in different approaches.

Specifically, the following aims are proposed (Scheme 2):

Chapter 2: Development of pH-, thermo-, and oxidation-responsive polymeric micro/nano-scale hydrogel particles as therapeutic carriers

Chapter 3: Development of pH- and thermo-responsive water soluble polymers as constituents for nano-scale layer-by-layer assemblies

Chapter 4: Development of pH-, thermo-, and oxidation-responsive macro-scale polymeric hydrogels as scaffolds for neural tissue engineering



Scheme 2 Overview of the dissertation research.

Chapter 2 Synthesis and Characterization of Micro/Nano-Scale Polymeric Hydrogel Particles with Multiple Responsiveness for Drug Delivery

Two articles presenting part of the results in this chapter have been published in Journal of Materials Chemistry B [(1) Tang S., Shi Z., Cao Y., He W. Facile aqueous-phase synthesis of multi-responsive nanogels based on polyetheramines and bisepoxide. Journal of Materials Chemistry B 2013; 1: 1628-1634. and (2) Tang S., Huang L., Shi Z., He W. Water-based synthesis of cationic hydrogel particles: effect of the reaction parameters and *in vitro* cytotoxicity study. Journal of Materials Chemistry B 2015; 3: 2842-2852.]. The first author (Shuangcheng Tang) performed the majority of experiments in this study. The corresponding author (Dr. Wei He) is advisor of Shuangcheng Tang and financially supported this work.

2.1 Introduction

Hydrogel particles of micro/nano-scale are promising for biomedical applications because of their characteristics including high water content, tunable sizes, crosslink structure and sufficient surface functional groups [38]. Due to their small sizes, the nano-scaled gel particles are able to freely penetrate tissues. The scaffold could be functionalized for immobilization of drug molecules, and the crosslinked network benefit the sustained drug release due to the spatial hindrance against diffusion. Surface moieties can be manipulated for cell targeting and resistance of rapid clearance by phagocytic cells. Endowed with aforementioned exceptional properties, micro/nano-gels have found

numerous potential applications in the biomedical fields, from delivery of therapeutics such as drugs [39], proteins [40], RNA and DNA [41, 42] to imaging [43] and biosensing [44].

Production of micro/nano-gels can be achieved with a number of methods, such as direct polymerization of monomers in heterogeneous colloidal environments, self-assembly of precursor polymers, coacervation crosslinking, and template-assisted nanofabrication [45-48]. Despite their continuous development, each of these methods has its inherent limitations. For example, the colloidal approach, either oil-in-water micro-emulsions or water-in-oil inverse micro-emulsions, involves the use of an organic solvent and the addition of stabilizing surfactants that require extensive post-production purification steps. Preforming a polymer for subsequent nanogel preparation can be time-consuming and often requires expertise on elaborate synthesis of well-defined polymer structures such as diblock, triblock, or hyperbranched structures. The template-based nanofabrication approach is commonly realized by photolithographic techniques or micro-molding methods. Although offering fine control over the micro/nano-gel shape and size, such an approach depends closely on high precision fabrication facilities and properties of replica molds.

To further facilitate the development of micro/nano-gel fabrication, a novel preparation method is presented in this chapter based on the thermally induced phase separation of polymers in step-growth polymerization. Our nanogel system is built on the reaction of a poly(propylene oxide) (PPO)-containing triamine, trade name Jeffamine T-403, with a bisepoxide, 1,3-butadiene diepoxide, in water, resulting in thermo-sensitive intermediates. In principle, the nanogel formation is initiated by the phase separation of

the in situ formed thermo-sensitive intermediate polymer into precursor particles at elevated temperature, followed by aggregation and further crosslinking of precursor particles through amine–epoxide reaction to yield robust nanogels (Scheme 3). Such a straightforward process suggests that tuning of the nanogel size distribution can be achieved by merely adjusting the concentration of the feedstock. While the use of thermo-sensitive polymers for nanogel preparation has been reported in the literature, none of them matches the facileness presented by our system. In comparison, most of the prior work used pre-synthesized polymers with thermoresponsive characteristics and stabilized the polymer colloids subsequently with chemical- or photo-crosslinking [46, 49, 50]. Recently, Yu and co-workers developed a temperature induced self-assembly and self-crosslinking method through disulfide bond formation for nanogel preparation [51]. Although it eliminates the use of any emulsion stabilizer, catalyst or additional crosslinking agent, it still requires presynthesizing a hyperbranched polymer and uses methanol as the solvent. Our approach is superior to Yu's approach in that it eliminates the need for any organic solvents and directly prepares the nanogels from monomers in a one-pot fashion.

In addition, majority of the reported nanogel systems prepared directly from polymerization of monomers are based on the free radical polymerization mechanism with vinylic monomers, including either conventional free radical polymerization or advanced living/controlled radical polymerizations such as atom-transfer radical polymerization (ATRP) and reversible addition–fragmentation chain transfer (RAFT) polymerization. Very few studies explored other polymerization mechanisms such as step growth polymerization [52].

Our study bridges this gap and expands the methodology tool kit available for nanogel fabrications. Furthermore, the nanogels prepared in this report are multi-responsive. As shown in Scheme 3, the resultant polymer consists of PPO, amine, and 1, 2-glycol groups, which results in nanogels being responsive to multiple stimuli, including temperature, pH, and oxidants. Compared with single-responsive drug carriers, multi-responsive vehicles provide better targeting efficiency and treatment efficacy [53]. Because in many diseases (e.g. cancer, pathological processes, diabetes, etc.), more than one physiological environment stimulus would change, particularly temperature and pH. Therefore it is of great interest to develop multi-responsiveness [54]. It is also worth mentioning that the use of water as the sole dispersant and the absence of additive/catalyst make this an environmentally friendly process.

To thoroughly examine the properties of gel particles and optimize the parameters for drug delivery, this preparation method was performed and characterized in several sections. First, a fabrication protocol based on Jeffamine 403 and 1, 3-butadiene diepoxide was determined for reproducible preparation of gel particles. The hydrodynamic size, ζ potential, and multi-responsiveness of obtained gel particles were carefully characterized. Second, the effects of reaction parameters on the particle properties were investigated for a better understanding of mechanism and guidance of tuning conditions of preparation. Third, cytotoxicity was evaluated via cell viability study of gel particles. Then drug loading function was studied using a model molecule Nile red. Investigation of release behavior was carried out in response to physiological environment stimuli.

2.2 Experimental Section

2.2.1 Materials

Jeffamine T-403 polyetheramine (JT403) and Jeffamine ED-900 polyetheramine (JED900) were kindly provided by Huntsman Chemical. 1, 3-Butadiene diepoxide (BDE) and polyethylenimine (PEI, branched, $M_n = 20,000$ g/mol) were purchased from Sigma-Aldrich. Hydrogen peroxide (30%) was purchased from Fisher Scientific. Nile red was kindly provided by Dr. Bin Zhao (Chemistry Department in UTK). All chemicals were used as received.

2.2.2 Micro/nano gel particles synthesis

In a typical preparation approach, the monomer JT403 (500 mg) was thoroughly mixed with deionized (DI) water (4 g) in a vial before adding BDE (139.4 mg) to reach a final monomer concentration around 15 wt%. The stoichiometric ratio of amino hydrogen to epoxide group in total was kept 2:1. The solution was then incubated without stirring in an isotherm water bath at 65 °C for 15 min (first session) to allow the epoxy-amine reaction to form the water soluble intermediate prepolymer. In the first session, the clear solution turned cloudy in 2 min suggesting the phase separation of the in-situ formed oligomers. The prepolymer solution was cooled using an ice bath until transparent before being diluted to a concentration of 0.5 wt% with DI water at room temperature in a flask, and returned to water bath at 65 °C to further react for 30 min (second session) without stirring. The diluted solution became translucent at the beginning of the second session indicating that phase separation of the obtained prepolymers. The solution appearance remained unchanged after being cooled down suggesting that the hydrogel particles formed in the suspension. After synthesis, the hydrogel particles were collected and

purified via repeated centrifugation at the speed of 21,000 RCF and dispersion in DI water.

For a comprehensive understanding of the reaction mechanism and influence of reaction parameters, monomers ratio, reaction time, reaction temperature, monomer concentration, and monomer composition were varied.

2.2.3 Characterizations

Hydrodynamic sizes and zeta (ζ) potentials of the synthesized hydrogel particles were determined using a Zetasizer Nano-ZS system (Malvern Instruments, Malvern, UK) with a fixed angle of 173°. The particles were dispersed in water at a concentration of 0.1 mg/mL and measurements were performed at 25 °C (all default hydrodynamic sizes are intensity sizes by zetasizer at 25 °C, unless otherwise stated). The data presented are the averaged values of at least three measurements. To obtain particle sizes at different temperatures, the samples were allowed to thermally equilibrate at the testing temperature for 30 min prior to data collection. The response of particles to pH was determined after 30 min equilibration and at least 10 min sonication. Scanning electron microscopy (SEM) images of the hydrogel particles were taken on a LEO 1525 Field Emission SEM (LEO Electron Microscopy, NY) at an accelerating voltage of 2 to 3 kV. The SEM samples were prepared by depositing a drop of particle containing solution onto a silicon substrate, dried, mounted on aluminum studs, and sputter-coated with gold to minimize sample charging during imaging. Transmission electron microscopy (TEM) study was performed using a ZEISS LIBRA 200 HT FE MC TEM. For preparing TEM samples, the purified particles were diluted to 4 mg/mL followed by 10 min sonication. Then the particle solutions were dropped on carbon-coated copper grids. After 2 min of incubation the

solutions were removed by filter paper. For sample staining, 0.5% uranyl acetate was dropped on the grid for 1 min before being drawn off from the edge of the grid with filter paper. The cloud points of the monomer JT403, JED900 and intermediate prepolymers were measured by monitoring the solution (2.5 mg/mL in DI water) transmittance at 400 nm with a Biomate 5 UV-Vis spectrometer. The cloud point was defined as the temperature corresponding to 50% transmittance. The nuclear magnetic resonance ^1H -NMR spectra were obtained using a Varian Mercury 300 NMR spectrometer (300 MHz) in deuterated water at the concentration of 15 wt%.

2.2.4 Degradation study via oxidation

The degradation of the gel particles were performed in the presence of hydrogen peroxide. The stock particle suspension was mixed with DI water and 30 % hydrogen peroxide to prepare a mixture containing 2.5 mg/mL particles and 5 % hydrogen peroxide. The optical density (OD) was monitored using a Biomate 5 UV-Vis spectrometer at the wavelength of 400 nm for 2 h at two-minute intervals. The particle size during the oxidation was determined and collected using a Zetasizer Nano-ZS system. The optical appearance of the solutions were obtained using a digital camera at the 0 h and 2 h during the degradation.

For the SEM images, the micro-sized gel particles were treated with 10 vol% hydrogen peroxide for 2 h. The SEM samples were prepared via freeze-drying the suspension droplets of oxidized particle on a silicon substrate. The samples were observed using SEM following the protocol mentioned previously in Section 2.2.3.

2.2.5 Nile red encapsulation and triggered release study

The gel particle suspension (2 mg/mL) was incubated at 65 °C for 20 min before being mixed with Nile red solution (1 mg/mL in acetone) at the volume ratio of 10:1. After agitation using a vortex mixer, 20 min incubation at 65 °C was performed followed by cooling using an ice bath. After being incubated overnight at 4 °C, the supernatant was saved for further study.

The release study was performed via changing the temperature, pH, or introducing oxidative stress in the Nile red containing particle solutions. Due to the limitation of the equipment, the temperature of the solution was varied from 0 °C to 40 °C at a 10 °C interval for temperature study. For pH study, the pH of the solution was first raised from 7 to 13, and was then reduced from 13 to 1 at an interval of 2 to investigate the response of Nile red containing particles. The oxidation study was carried out in the presence of 1 vol% hydrogen peroxide for 16 h. The fluorescent intensity of the Nile red was monitored to determine the potential release of the dye. The peak wavelength and peak intensity of loaded Nile red were determined using a Perkin Elmer LS55 luminescence spectrometer with the excitation at the wavelength of 550 nm.

2.2.5 *In vitro* cytocompatibility study

In vitro cytotoxicity was examined with macrophages. A subclone of murine derived RAW264.7 macrophages (kindly provided by Dr. Tim Sparer at The University of Tennessee) were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in 100-mm tissue culture treated petri dishes. Cell cultures were maintained at 37 °C in a 5% CO₂ balanced-air incubator and the culture medium was changed every 2 to 3 days.

Confluent cells were detached with a scraper, pelleted and resuspended in fresh medium for cytotoxicity assays.

RAW264.7 macrophages at a density of 1×10^5 cells/well in 100 μ L medium were seeded to a 96-well plate and allowed to attach and grow for 20 h. Culture medium was then aspirated and replaced with DMEM supplemented with various types of hydrogel particles at the indicated concentrations. After 4 h of incubation in the presence of hydrogel particles, the mitochondrial dehydrogenase activity of cells was assessed using a water soluble tetrazolium salt (WST-1) assay (Roche Applied Science, USA) in order to evaluate cell viability. Briefly, 15 μ L of WST-1 reagent in 150 μ L culture medium was added to each well to displace the particle-containing DMEM. The plate was incubated at 37 °C for 2 h followed by 1 min shaking. Supernatants (100 μ L) containing the water-soluble reaction product formazan dye were transferred to a new 96-well plate, and the absorbance at 440 nm was obtained using a BioTek Synergy 2 Multi-Mode Microplate Reader (BioTek, USA) against a blank control for background correction. Positive control cells were cultured without any treatment, and negative control cells were treated with cationic polymer PEI. The viability of the cells exposed to hydrogel particles was expressed as a percentage to the positive control. The effects of particle types and concentrations were statistically analyzed using Student's *t*-test. Data were considered to differ significantly when $p < 0.05$.

For cell imaging, macrophages were seeded on silicon substrates and the cell morphology after cytotoxicity assay was examined under SEM. Cell layers were fixed, dried, and gold sputtered prior to imaging, following procedures as described previously [55]. Secondary electron images were acquired on a LEO 1525 Field Emission SEM

(LEO Electron Microscopy, NY) at accelerating voltages of 2 to 5 kV. Changes in cell morphology after hydrogel particle treatments were also observed in parallel with the above cytotoxicity study using a Zeiss Observer A1 inverted optical microscope.

2.3 Results and Discussions

2.3.1 Characterization of particles based on JT403 and BDE

The hydrogel particles based on JT403 and BDE were prepared following the aforementioned procedure in the experimental section. The monomer stoichiometric ratio of amino hydrogen to epoxy was 2: 1. Under this ratio, the number of moles of primary amine groups ($-NH_2$) equals the number of moles of epoxy groups, which corresponds to a monomer molar ratio of JT403 to BDE at 2: 3.

In the first session, about 4 mL aqueous solution containing 15 wt% total monomers with completely colorless and transparent appearance became white cloudy in 130 seconds during the incubation at 65 °C. The transition of opaqueness can be explained using the phase separation behavior of the *in-situ* formed oligomers. The JT403 aqueous solution does not turn cloudy at 65 °C due to its cloud point at 76 °C below which no phase separation occurs. But in this reaction, a step-growth polymerization of JT403 and BDE took place via ring opening reaction forming oligomeric chains. The chain length increased as the time went on. For the polymers of same composition, the solubility decreases as the increase of chain length [56]. Therefore the growth of polymer molecule weight reduced solubility of chains in water and resulted in decrease of cloud point. When the cloud point of the *in-situ* formed intermediate was below 65 °C, phase separation occurred and the solution exhibited an opaque appearance. The cloudy

solution appeared homogeneous at the reaction temperature, and then became colorless and transparent after being cooled using an ice bath. It is speculated that the intermediate product of the first session is not crosslinked. After being diluted 30 times to 0.5 wt%, in the second session, the solution was incubated at 65 °C for 30 min. In the initial 30 seconds of incubation, the mixture turned turbid gradually as warming up. In the whole second session the turbidity of the solution maintained. And after the reaction, the mixture was cooled down to room temperature but the cloudiness remained unchanged.

The average hydrodynamic size of the crude products (unpurified suspension) is around 110 nm (Figure 5b). The suspension was homogenous and stable at room temperature for at least a week without any visible precipitates or aggregates. The purified particles exhibited an average hydrodynamic size about 410 nm with a polydispersity index (PDI) at 0.18 (provided by the Zetasizer software) (Figure 5b). The apparent average hydrodynamic size of products increased from 110 nm to 410 nm after the purification due to the removal of non-crosslinked oligomers. The size change is reasonable considering that the crude products contain oligomers, non-crosslinked branched polymers, and particles varying in sizes. It is estimated that the gravimetric fraction of the collected particles is less than 5 wt% of the raw materials (about 20 mg over 640 mg). This small portion may be negligible in the size distribution curve of the crude product because of the overwhelming signal intensity of fine size products. But size of this portion can be observed when the majority of the raw materials was removed showing a drastic change in apparent size.

In addition to the hydrodynamic sizes determined using zetasizer, the size and morphology of dehydrated particles were investigated using SEM and TEM (Figure 6).

(a)

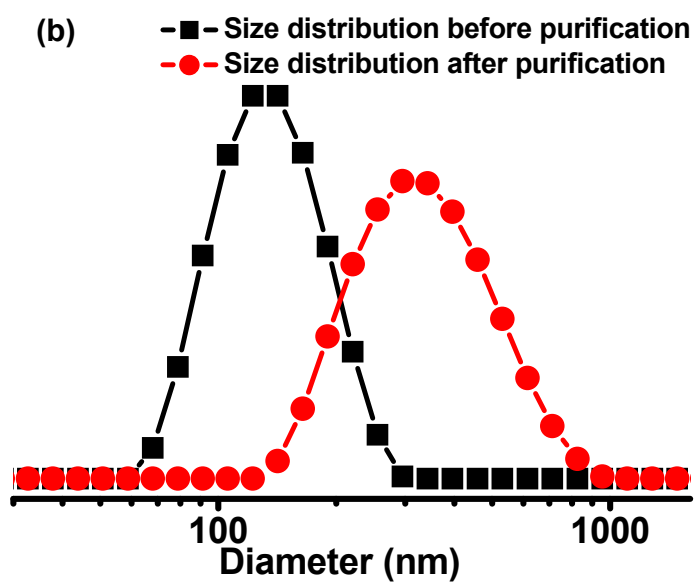


Figure 5 (a) A photomicrograph of a purified suspension of particles based on JT403 and BDE reaction. (b) Size distribution of the particle suspension before and after purification.

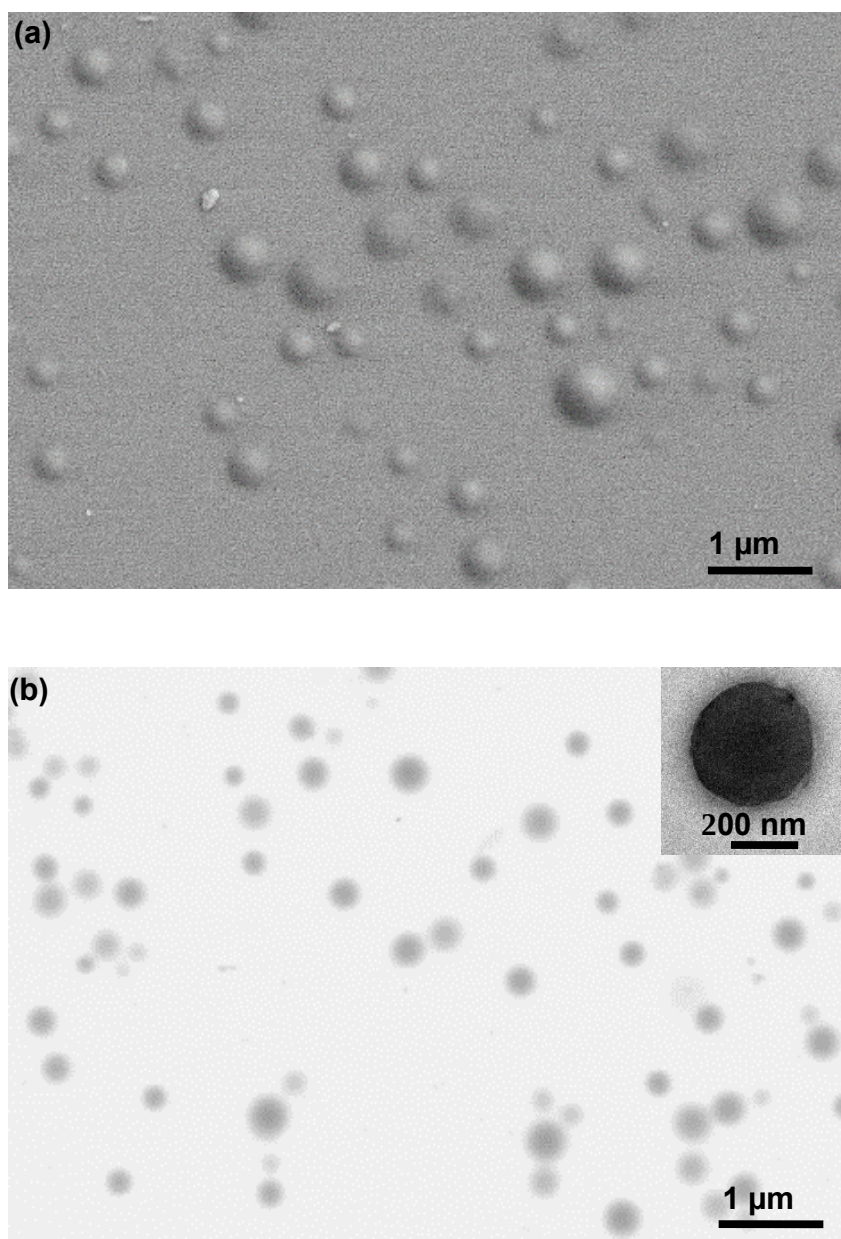


Figure 6 The sizes and morphology of dehydrated particles based on JT403 and BDE were characterized by (a) scanning electron microscopy (SEM) and (b) transmission electron microscopy. The particle showed in inset was stained by uranyl acetate.

The SEM image (Figure 6a) demonstrated that unlike rigid spherical particles based on polymers such as polystyrene, the ether-amine containing particles exhibited their soft nature judging by the bulging appearance of the air-dried sample. The size and size distribution are obtained from TEM images which provided better contrast than SEM images for defining the edge of particles. The average diameter of dehydrated samples is 191 nm with a standard deviation of 60 nm. The average diameter of particles shrunk about 53% after being dried, while the size distribution of the particles were consistent before and after dehydration. Furthermore, it can be speculated that the particles are structurally homogeneous based on the TEM image (Figure 6b inset) of the particle stained with uranyl acetate.

The particle is composed of ether-amine containing backbones, so the charge density is one of the important characteristics of the vehicle for biomolecules. For the colloids, the theoretical interface of shear (or slipping plane) is defined as an imaginary surface which is close to the solid surface containing stationary fluid [57]. When an electric field is applied, the charged particles move along with a certain quantity of the surrounding liquid within the slipping plane. The difference of electric potential between the slipping plane and a point in the bulk fluid is named ζ (zeta) potential. As shown in Figure 7, the surface charges of the particles based of JT403 and BDE are positive due to the presence of amine groups with the average ζ potential being around 65 mV.

The chemical structural changes during the reaction were determined using ^1H NMR. Briefly, JT403 and BDE were mixed with D_2O for ^1H NMR monitoring. The concentration of total reactants became 13.5 wt% due to the increased molar mass of the solvent from 18 g/mol to 20 g/mol. The ^1H NMR spectra were obtained at room

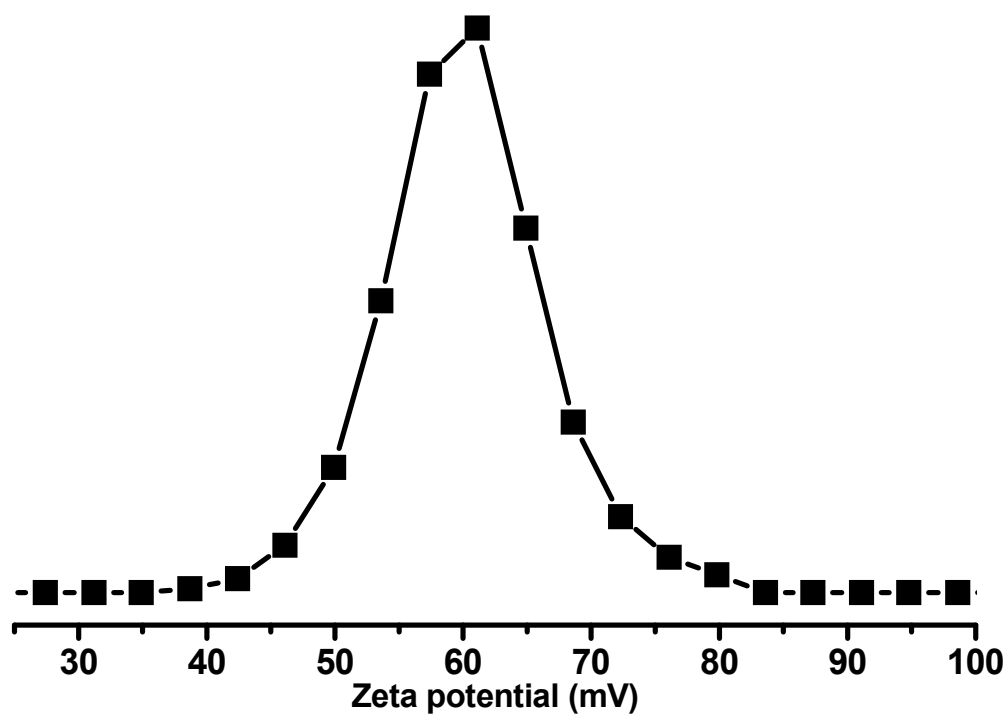


Figure 7 Charge density distribution of particles based on JT403 and BDE.

temperature instead of 65 °C for two reasons. First, the reaction temperature (65 °C) was not feasible with the NMR instrument available for this study. Second, the ¹H NMR detection demands well dissolution of solutes in solvents so the phase separation should be avoided. The mixture was incubated overnight at room temperature in order to form soluble oligomers. The spectra of JT403, mixture of the reactants before and after overnight reaction were collected and demonstrated in Figure 8. The protons of the BDE assigned to the peaks at chemical shift 2.7 ppm (quartet), 2.8 ppm (multiplet) and 2.9 ppm (multiplet) disappeared after the reaction. Instead, the bonds formed between amino and epoxide group as the overlapped peaks appeared around 2.7 ppm proving the ring-opening reaction.

2.3.2 Responsiveness of particles based on JT403 and BDE

2.3.2.1 Thermoresponsiveness

As a crosslinked network composed of thermosensitive polyetheramine backbones, the hydrogel particles showed temperature-responsive behavior, such as swelling-shrinking in hydrodynamic sizes. As shown in Scheme 3, the hydrogel particles, containing blocks of PPO and PEO chains, are structurally very similar to the pluronic polymers, a well-known family of temperature responsive materials [58]. A zetasizer was used to detect the size change with disposable plastic cuvettes. The upper limit of the temperature testing was 45 °C due to the poor thermal stability of the plastics cuvettes. A decrease in the hydrodynamic size of the particles based on JT 403 and BDE was observed when the temperature was raised from 0 °C to 45 °C (Figure 9a). On the contrary, the particles swelled during the cooling process as shown in Figure 9b. Between the upper and lower limit of testing temperatures, the average diameter indicated a

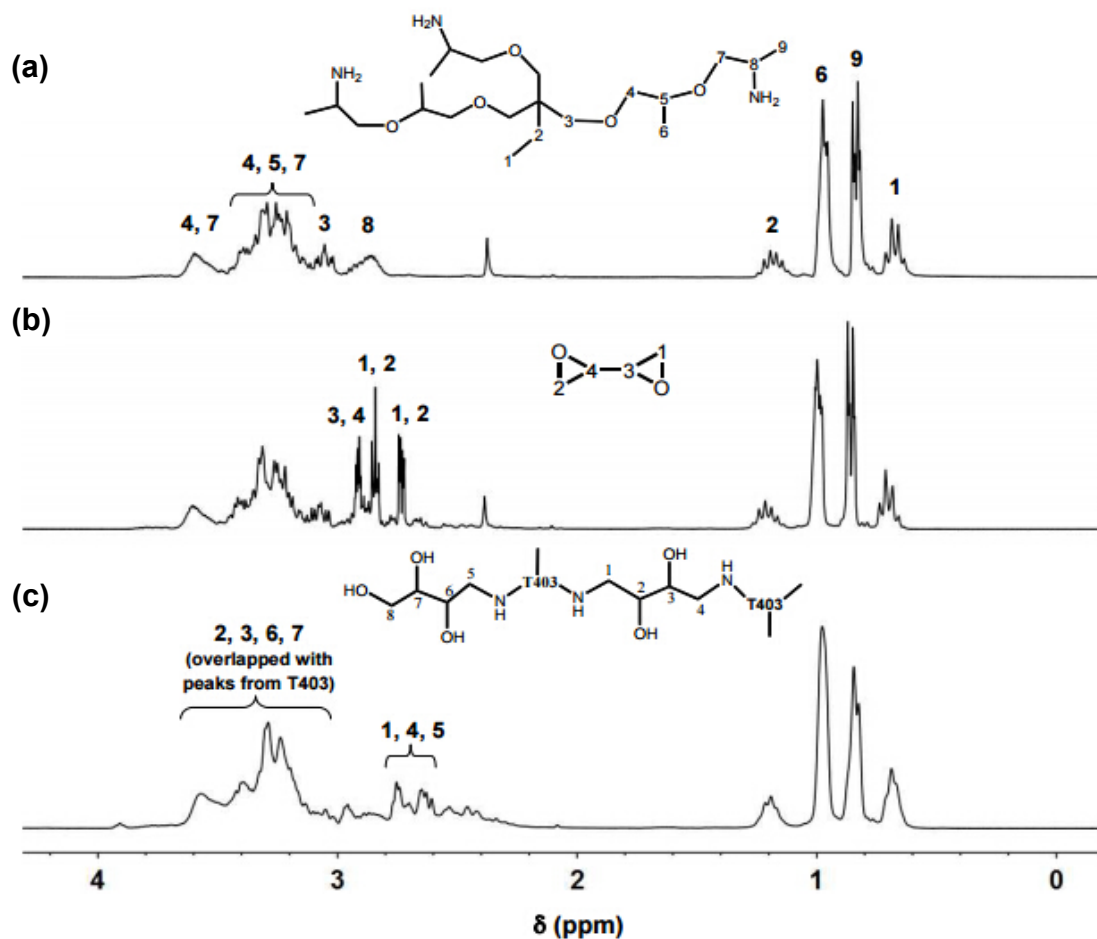


Figure 8 ^1H NMR spectrum of JT403 (a), and mixture of JT403 with BDE at time 0 h (b) and time 24 h of reaction (c) in D_2O .

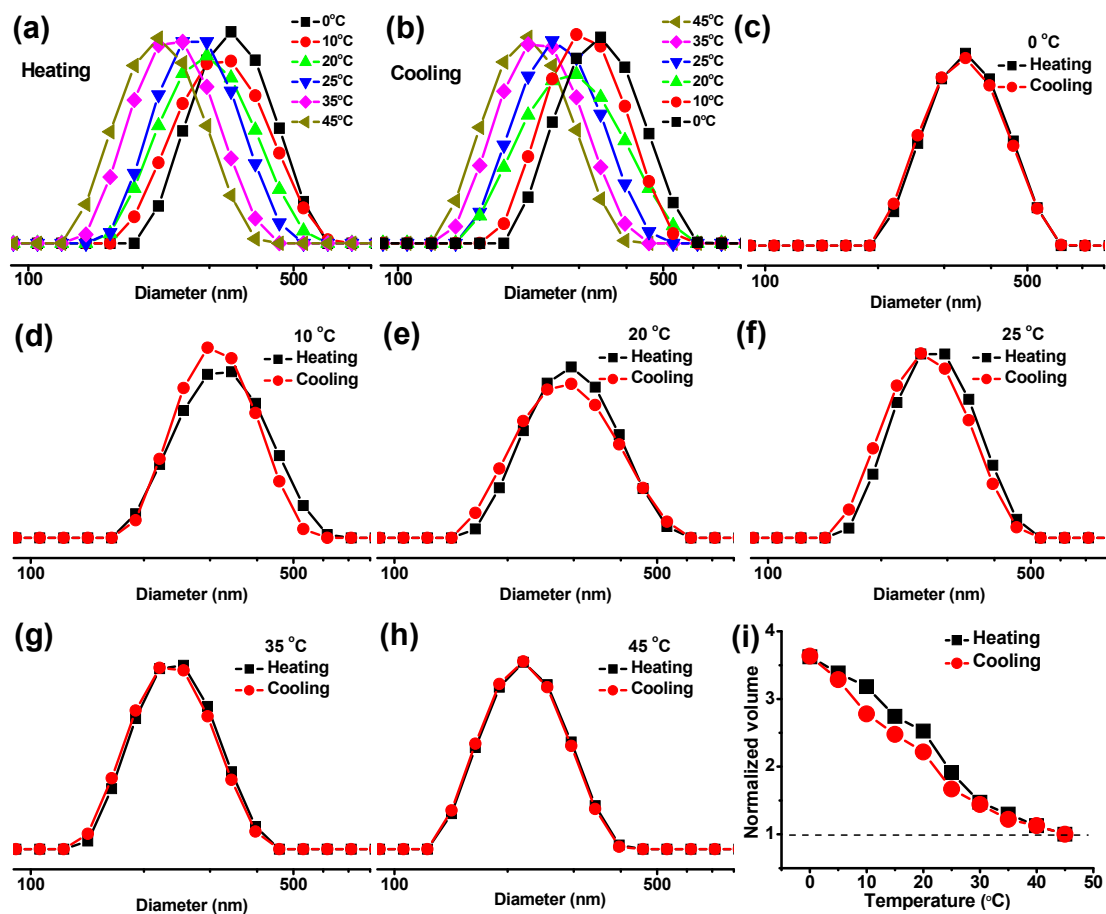


Figure 9 Size distributions of particles based on JT403 and BDE at different temperatures during (a) heating and (b) cooling were collected using zetasizer. The size distributions of particles based on JT403 and BDE during heating and cooling process were compared respectively, at (c) 0 °C, (d) 10 °C, (e) 20 °C, (f) 25 °C, (g) 35 °C, and (h) 45 °C. And (i) the volume of particles were normalized and summarized as a function of temperature.

difference of 173 nm (i.e. 73% change in volume). Such a temperature responsiveness can be easily interpreted through the hydration–dehydration process [59]. At low temperatures, the particle is in its hydrophilic state, well-hydrated with trapped water through hydrogen bonding; while at high temperatures, the nanogels collapses into its hydrophobic state, leading to disruption of hydrogen bonds with water molecules and consequently dehydration of the particle.

It was noticed that the sizes of particles were consistent at 0 °C after cooling as well as those at 45 °C after heating. But except for 0 °C and 45 °C, hysteresis was observed between the heating and cooling processes in hydrodynamic sizes. As shown in Figure 9c-h, the size distribution curves were compared in detail at different temperatures respectively. Obvious dynamic lag in hysteresis were noticed from 10 °C to 25 °C while the greatest variance of 20 nm in size was observed at 10 °C probably due to the hysteretic conformation adopting of polymer chains. But at each temperature, the polydispersity and size distribution profile are not drastically different between the heating and cooling courses.

The volumes of particles were normalized against the average volume at 45 °C and were then summarized in Figure 9i as a function of temperature. The volume of the particles at 0 °C is about 3.65 fold larger than that at 45 °C. It was observed as well that the largest size transition occurred between 15 and 25 °C. The thermal transition profile of the particles based on JT403 and BDE relates to the average molecular weight between neighboring crosslink points, or the flexibility of the network. The details will be discussed later with particles obtained under different reaction conditions.

2.3.2.2 Responsiveness toward pH

The network of the particles is composed of polyetheramine with abundant amino groups. So it is expected that the hydrogel particles are responsive to pH considering the nature of amino groups [60]. At low pH, the basic amino groups are protonated (ionized), leading to swelling of particles because of the electrostatic repulsion among these positively charged groups as well as the rise of internal osmotic pressure. As the pH increases, deprotonation of the amino groups would reduce the hydrophilicity and electrostatic repulsion within the particles. The decrease of hydrophilicity can induce the folding of chains, the increase of χ_1 , and resulting increase of refractive index difference between the particles and the surrounding water. The opaqueness variance of solutions at different pH was observed (Figure 10a). It is interesting that as the particles became less hydrophilic in the basic environment, the aggregation and precipitation occurred. As shown in Figure 10a, in 2 h at pH 10 the precipitates are visible, and in 17 h the solution turned clear with obvious sediment. Therefore, to minimize the influence of aggregation, sonication of 10 min was performed to all the particle solutions before sizes measurement using zetasizer. Sizes and distributions demonstrated in Figure 10b indicated that the particle sizes decreased from 490 nm, 470 nm, 450 nm, to 430 nm as the pH increased from 2, 4, 7, to 10, respectively. The polydispersity of particle size at pH 10 is wider than those at other pH due to the aggregation. In addition, the volumes normalized against the size at pH 7 were summarized in Figure 10c. It was found that the volume of the particles at pH 2 is about 3.6 fold larger than the apparent volume at pH 10.

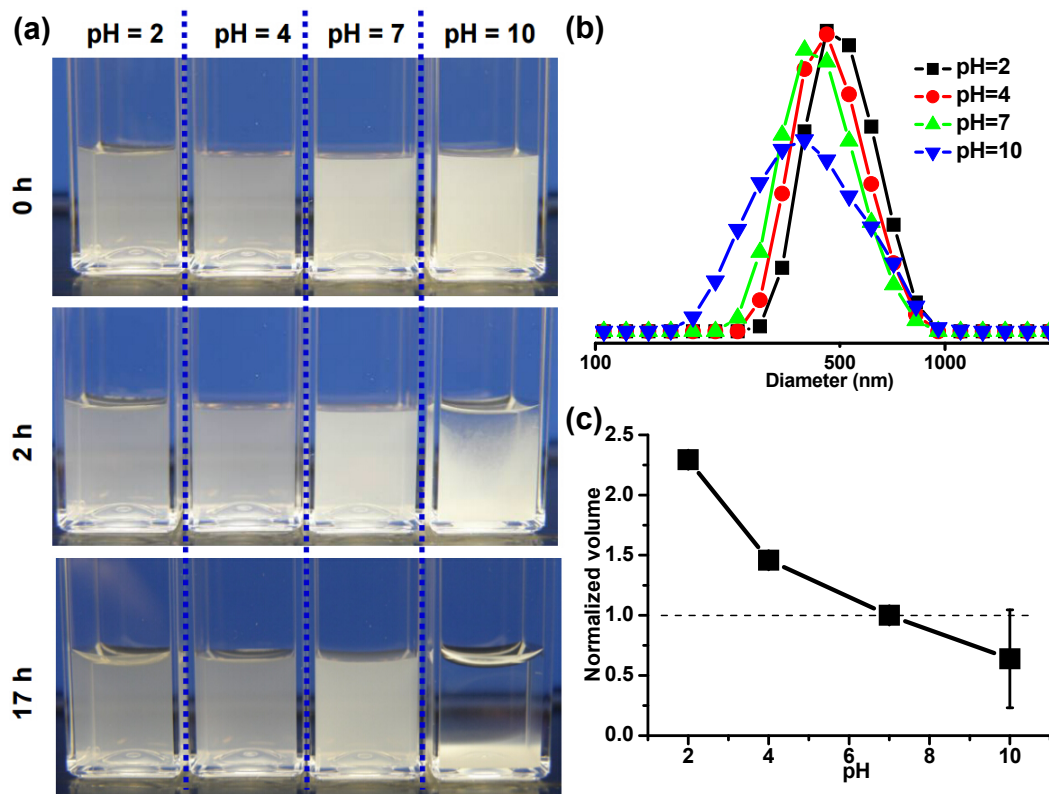


Figure 10 (a) Visual observation of the particle solutions at different pH as a function of time. The particle sizes and distributions (b) were determined using zetasizer. The volumes of particles were normalized against average volume at pH 7 for the summary as a function of pH (c).

2.3.2.3 Responsiveness toward oxidative stress

In addition to the responsiveness toward temperature and pH, the biodegradability is critical for the drug delivery system [61]. First, the degradation of vehicles would provide assistance for a thorough release of drug *in vivo*. Second, long-term retention of a non-degradable foreign material would cause chronic inflammation and subsequent rejection response from the immune system. Therefore it is crucial to incorporate degradability to allow the particles to decompose after the drug being delivered. Furthermore, to avoid secondary cytotoxicity of the vehicles, products generated during the course of degradation should be nontoxic, absorbable, and could be eliminated via normal metabolic pathways.

Here, H_2O_2 was used as the oxidant, a biologically relevant oxidant typical of inflammation and foreign body reaction [62, 63]. As shown in Scheme 3, the ring opening reaction of BDE introduced 1, 2-glycol groups into the backbone of the network. The oxidative cleavage of 1, 2-glycol units in the chain occurs in the presence of hydrogen peroxide (Figure 11) [64].

The visual observations of particle suspension before and after oxidation in the presence of hydrogen peroxide were demonstrated in Figure 12a. The cloudy suspension turned completely transparent after 2 h treatment with 5 vol% H_2O_2 . Turbidity measurements were used to follow the oxidation of the particles. The particle suspension showed a gradual decreasing trend of absorbance as a function of UV-vis wavelength (Figure 12b). The OD at 400 nm was applied to monitor the process of oxidation. A clear decrease in the optical density (OD) was observed (Figure 12c) by UV-vis spectroscopy. The reduced optical density can be attributed to the decrease in the refractive index

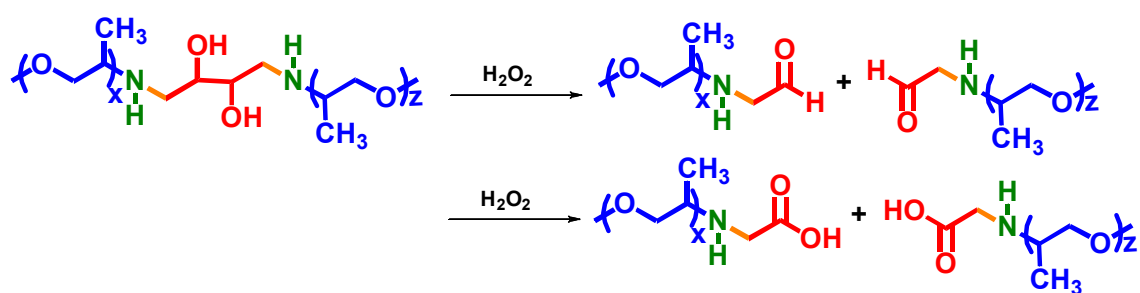


Figure 11 Scheme of the breakage of 1, 2-diglycol linkage during the oxidation in the presence of hydrogen peroxide.

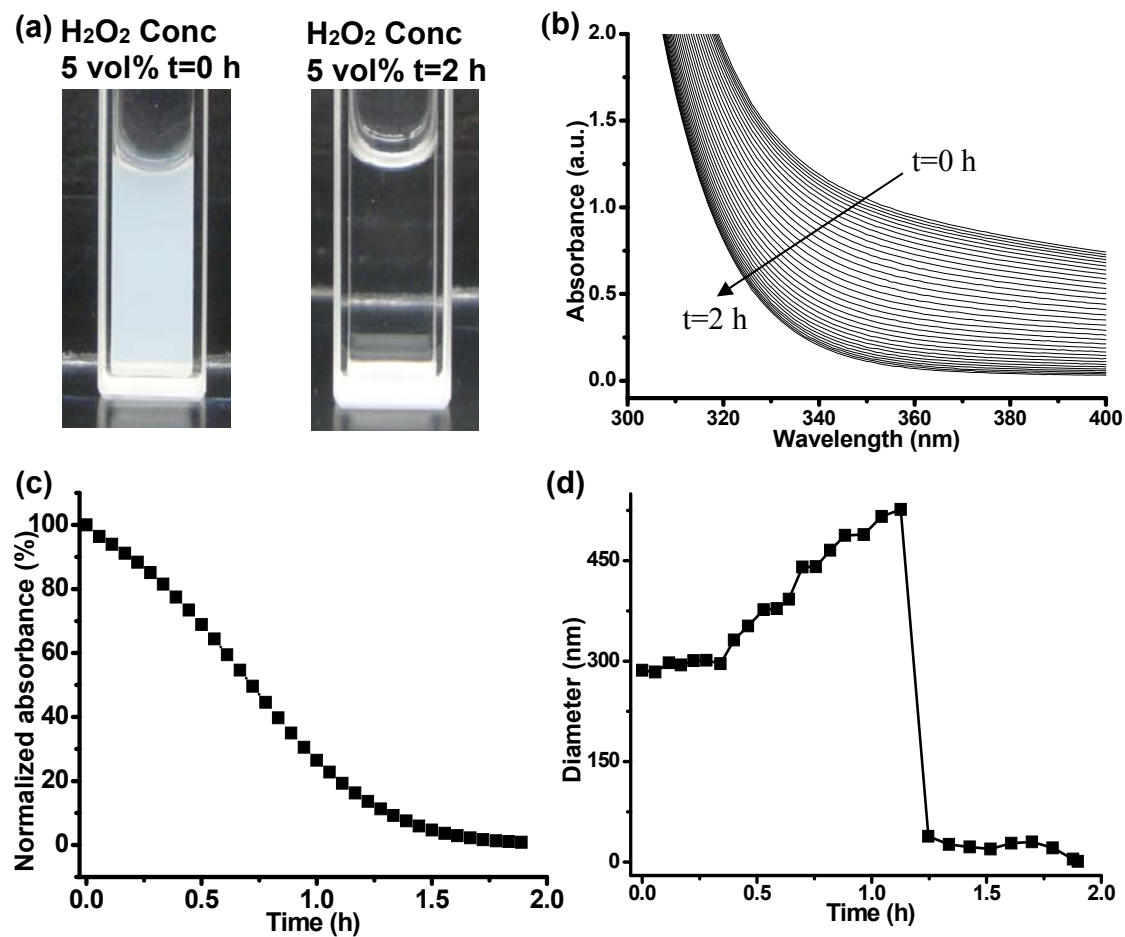


Figure 12 Visual observation (a) and UV-Vis spectra (b) of particle suspension during 2 h oxidation with hydrogen peroxide at 5 vol%. Normalized absorbance at 400 nm (c) were summarized as a function of time. The hydrodynamic sizes (d) were collected in the presence of 10 vol% hydrogen peroxide using zetasizer.

difference between the particles and the surrounding water, resulting from oxidation induced fragmentation of the particles. In addition, the degradation of the particles would result in a decrease in molecular weight, which in turn would change the scattering cross-section and lead to a reduction in turbidity.

DLS measurements were also used to monitor such a response in the presence of 10 vol% hydrogen peroxide. DLS tests show a size increase within the first hour, followed by a sudden loss of integrity (Figure 12d). This could be attributed to oxidative breakage of the 1, 2-glycol linkage (Figure 11), reducing crosslinking points within the particles, and consequently resulting in a gradual swelling of the particles. Eventually, the crosslinking points were disrupted to an extent that the gel particles broke into fragments.

The SEM images obtained from oxidation of micro-sized gel particles exhibited a corresponding evidence that the particles swelled (Figure 13). It is observed that after 1 h treatment with hydrogen peroxide, the particles appeared as bulging domains with debris but the edge of individual particles can still be determined (Figure 13b). After 2 h oxidation, there was only fragments without defined shape (Figure 13c).

2.3.3 Effects of reaction parameters on particle formation and properties

2.3.3.1 Monomer ratios

The monomer stoichiometric ratio of amino hydrogen to epoxy was varied as 3:1, 2:1, 1:1, and 1:2, to investigate influence toward formation of particles. It was found that only at the molar ratio of 2:1, individual particles without aggregation were successfully obtained. At this ratio, the molar mass of primary amine groups ($-NH_2$) equals the molar mass of epoxy groups, correspondingly the monomer molar ratio of JT403 to BDE is 2:3.

It is known that, after the reaction between the primary amine and epoxy, the

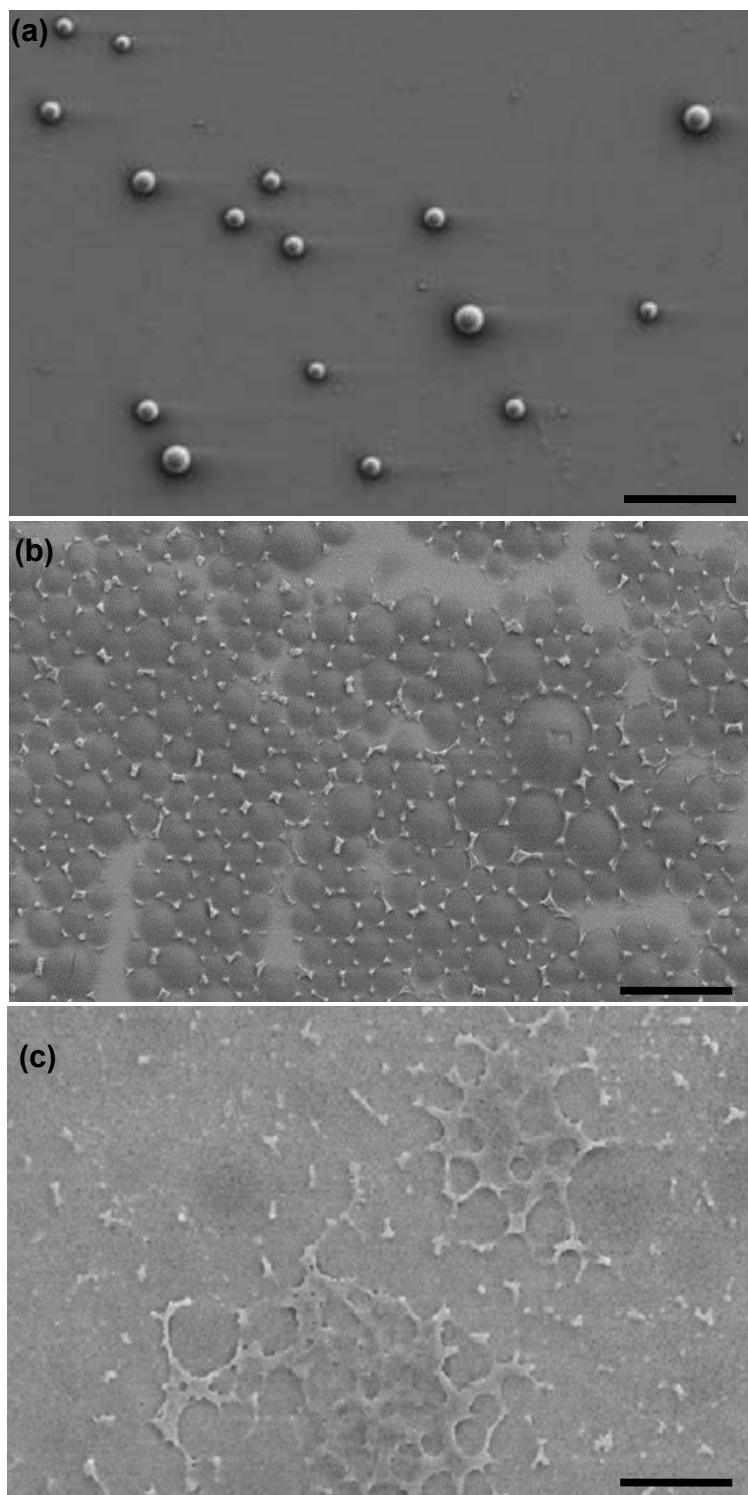


Figure 13 SEM images of particles before (a) and after treatment with 10 vol% H_2O_2 for 1 h (b) and 2 h (c). Scale bar = 10 μm .

primary amine will get converted into the secondary amine, which is less chemically reactive compared to the primary amine (Figure 14). The reaction kinetic rate constant of epoxy with primary amine group (k_1) is much higher than that with secondary amine group (k_2) [65], and $k_2/k_1 < 0.3$ for equal reactivity of amino hydrogens. When the stoichiometric ratio is 3:1 (or say 1.5: 1 for primary amino hydrogen to epoxide), the amino group is excessive by about 50%. For a copolymerization of multi-functional monomers, gelation point can be estimated using the stoichiometric ratio, concentrations of all reactants [66]. But for the stoichiometric ratio of 3:1 in our system, there is no gel point at 15 wt%. It is observed that the clear solution turned cloudy during the reaction at 65 °C suggesting formation of oligomers.

But the solution became transparent at room temperature supporting the non-existence of gels (Figure 15a). It could be explained that the cloud point of obtained oligomers is between room temperature and 65 °C. So the phase separation did not occur at room temperature but took place at the reaction temperature.

The overall synthesis conditions adopted in our studies favor the reaction between the primary amine and epoxy for gel formation, considering that the resultant secondary amine from JT403 is sterically hindered and exhibits low reactivity. Therefore, when the ratio of amino hydrogen to epoxy is 1:1 and 1:2 respectively, the reactive primary amine hydrogen is in fact stoichiometrically in shortage against epoxy groups. Excess epoxy would then end-cap the Jeffamine molecules and the crosslinking reaction cannot be achieved thoroughly, leading to the irregular morphology of resultant products shown in Figure 15c and d.

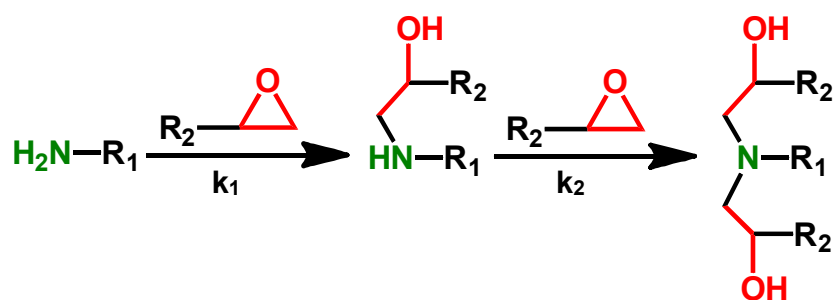


Figure 14 Scheme of two-step reaction between the primary amino group and epoxy group.

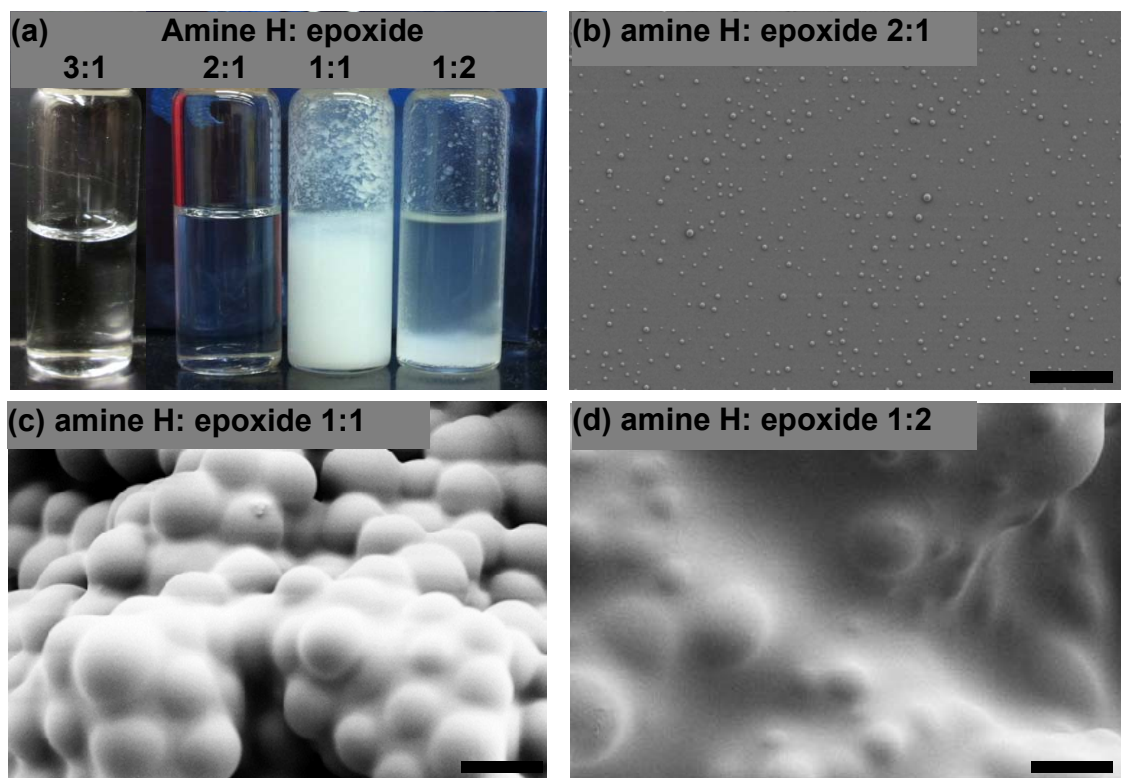


Figure 15 (a) Visual observation of solutions obtained from the first session at different monomer ratios, and scanning electron microscopy images of the reaction products based on amino hydrogen to epoxy stoichiometric molar ratio of (b) 2:1, (c) 1:1 and (d) 1:2. Scale bar is 10 μm .

2.3.3.2 Reaction time

As epoxy–amine chemistry based polymerization follows the step-growth mechanism, the influence of reaction time on particle formation was investigated. The reaction time for the first step was varied from 5 min to 20 min, while the second step was kept constant at 30 min. The reactant concentration was 15 wt% and reaction temperature was 65 °C. Spherical hGPs were formed for all conditions except the 20 min group, where a large chunk of bulk hydrogel was obtained after the first step. For the potential mechanism underlying the bulk gel formation in the 20 min group, one possibility could be phase separation of the formed prepolymers followed by coacervation and further network crosslinking beyond the gelation point. The size distribution of the resulting hGPs was characterized by zetasizer and the result is shown in Figure 16a as well as summarized in Table 1. Intuitively, one would expect the particles to grow in size as the reaction time prolongs. Yet, an inverse correlation was observed between the particle size and the reaction time of the first session. With the reaction time varied between 5 min, 10 min, and 15 min, the average hydrodynamic diameter of the particles decreased from 820 nm (hGP7), to 600 nm (hGP8), and to 410 nm (hGP9) (Table 1), respectively. Such a difference could be explained by the thermosensitive behavior of the resulting prepolymers. The formation mechanism of hydrogel particles for this particular system is postulated to be secondary aggregation of precursor nanoparticles resulting from the intermediate thermosensitive polymers produced *in situ* [67]. Since the intermediate polymers were formed by stepwise polymerization of epoxy with amine, it is expected that the polymer molecular weight will increase as a function of time. Although the intermediate polymers were not directly

Table 1 Synthesis conditions and physical properties of the resulting gel particles

Sample	First Step Conditions ^a			Hydrogel Particle Properties		
	<i>T</i> (°C)	<i>C</i> (wt.%)	<i>t</i> (min)	<i>D_h</i> (nm) ^b	<i>ζ</i> -potential (mV)	Swelling ratio ^c
hGP1	55	15	10	1000 ± 170	+50 ± 5	8.0
hGP2			15	720 ± 120	+60 ± 6	5.0
hGP3			20	600 ± 100	+71 ± 7	4.2
hGP4			25	macrogelation		
hGP5	65	5	15	790 ± 380	+47 ± 4	17.6
hGP6		10	15	510 ± 180	+49 ± 4	6.4
hGP7		15	5	820 ± 150	+56 ± 5	18.5
hGP8			10	600 ± 200	+61 ± 6	4.6
hGP9			15	410 ± 130	+66 ± 6	3.4
hGP10			20	macrogelation		
hGP11	75	15	5	440 ± 50	+48 ± 6	5.7
hGP12			10	230 ± 25	+51 ± 5	2.7
hGP13			15	macrogelation		

^aReaction conditions for the second step kept consistent: C% 0.5 wt.%, t 30 min;

^bIntensity average diameter obtained from DLS measurements in DI water at 25 °C;

^cSwelling ratio determined as $V_{0\text{ }^{\circ}\text{C}} : V_{45\text{ }^{\circ}\text{C}}$;

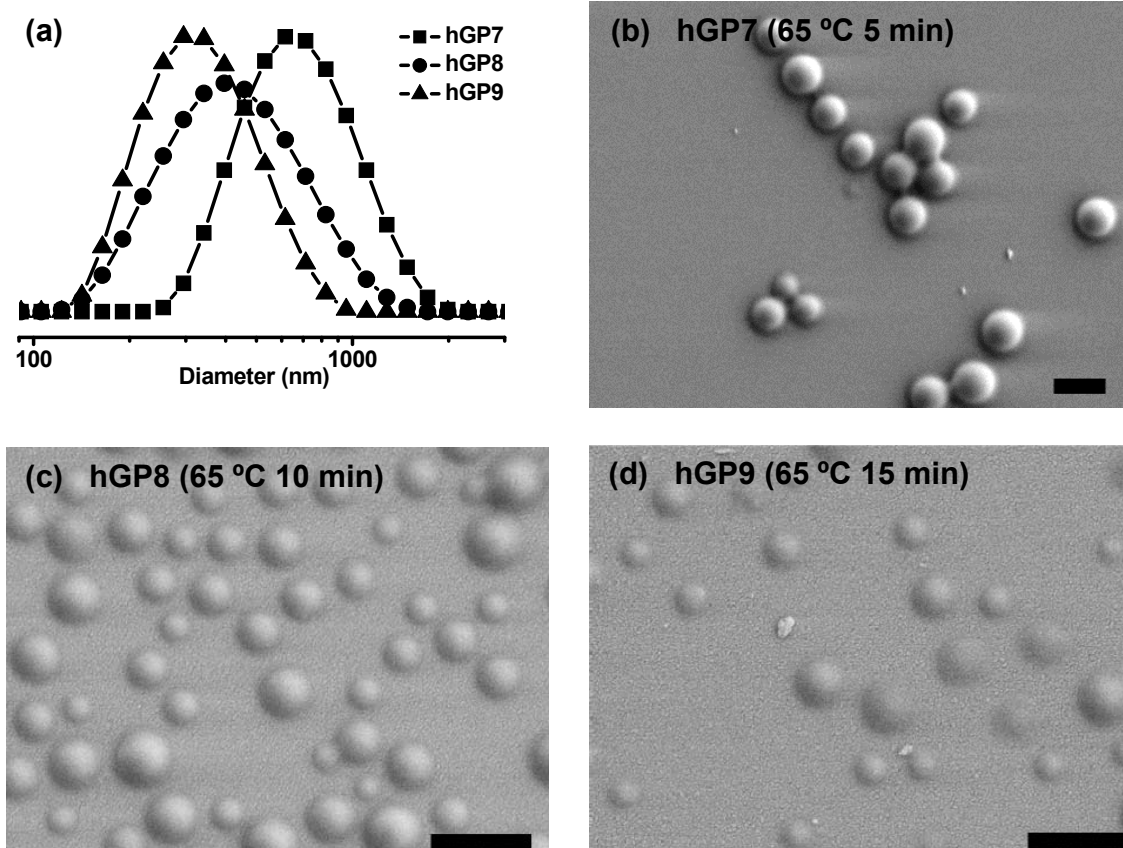


Figure 16 Effects of step one reaction time on hGP formation. (a) Representative intensity-averaged hydrodynamic size distribution of hGPs determined by DLS. (b–d) Scanning electron micrographs of hGPs. Scale bar is 1 μm .

isolated from the reaction mixture, polymer growth dependence on reaction time was evident from the cloud point measurements of the reaction solutions at the end of the first session. The monomer JT403 displayed a cloud point temperature of 76.5 °C (determined as the temperature at which the solution show 50% transmittance). As shown in Figure 17a, the cloud point temperature for the solution reacted for 5 min was 44 °C, significantly lower than that of the original monomer JT403, and it further decreased to 27 °C when the reaction time extended to 15 min. Such a decrease in cloud point temperatures suggests that the intermediate polymers were indeed formed and grew as the reaction time increases, since solution phase transition temperatures of many polymers exhibiting thermosensitive behaviors are known to be inversely dependent on polymer molecular weights [68-73]. As step-two reaction took place at 65 °C, higher than the cloud points of these intermediate polymers, it led to phase transition from well-solvated polymer chains (clear solution) to thermally induced dehydration and collapse of polymer chains into globules/aggregates (turbid solution). The sizes of the intermediate globules/aggregates were measured by DLS, and the results (Figure 17b) showed that the hydrodynamic sizes were 560, 450, and 220 nm for products collected after 5, 10, and 15 min reaction of step one at 65 °C, respectively. Therefore, the final hGPs (i.e., hGP7-9) obtained from the secondary aggregation reaction of these precursor nanoparticles exhibited a similar size trend as shown in Figure 16a. The inverse relationship between particle size and reaction time of step one was also observed for other reaction temperatures (i.e., 55 and 75 °C, Table 1).

The morphology of the particles was studied by SEM. As shown in Figure 16b–d,

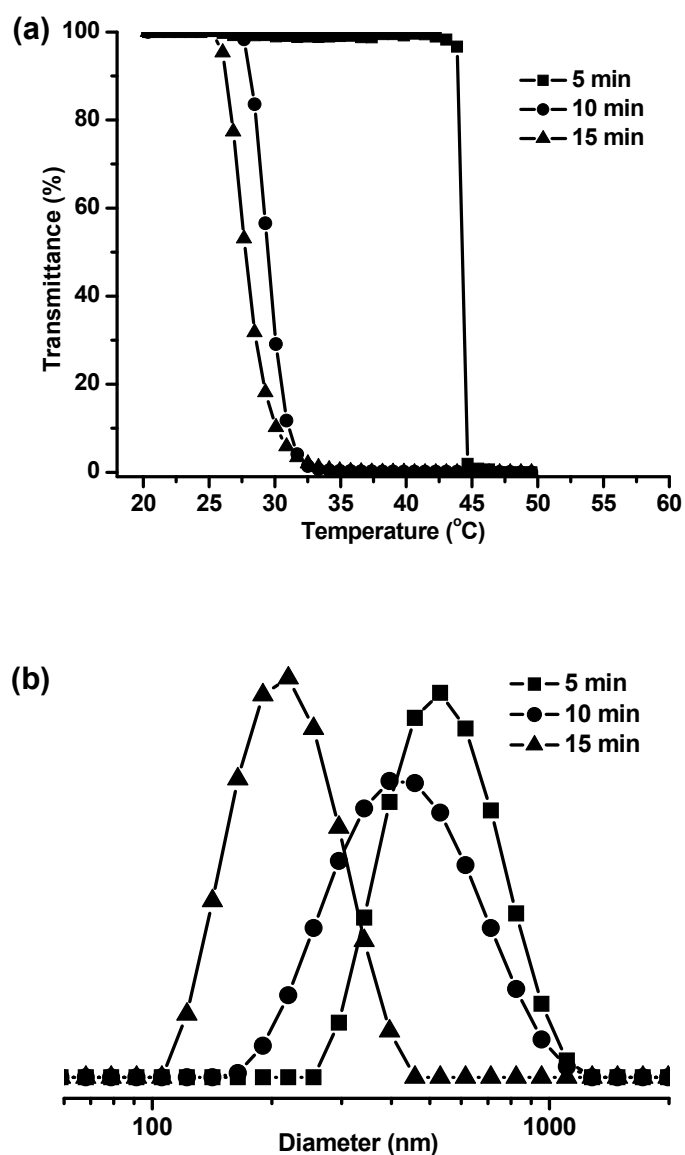


Figure 17 Solution properties of the reaction mixtures obtained after reacting for various durations (5, 10 and 15 min) in step one. (a) Solution optical transmittance as a function of temperature. (b) Intensity averaged hydrodynamic size distribution at 45 °C.

regardless of the reaction times, the resultant particles were round with a smooth surface. The average particle sizes measured from the SEM images were 735, 415, and 360 nm for hGP7, hGP8, and hGP9, respectively. These values were lower than the corresponding DLS values, due to the fact that these particles were dehydrated prior to SEM analysis. Nevertheless, the trend of size dependence on reaction time observed based on the SEM results was consistent with that obtained from the DLS measurements. Besides affecting hGP sizes, the reaction time also had a significant impact on the particle morphology. The SEM micrographs showed that hGP7 particles appeared to be much more spherical Figure 16b, whereas such three-dimensionality was drastically reduced for hGP8 particles (Figure 16c) and even more so for hGP9 particles (Figure 16d).

The morphological difference among these hGPs could be partially explained by the amount of precursor particles that eventually aggregated and formed the final hGPs. Considering the step-growth nature of the reaction, the solution mixture obtained after 5 min of step-one reaction would contain a much larger amount of low molecular weight prepolymers, as compared to that collected after 10 min step-one reaction. For the 15 min reaction, the mixture would mainly consist of high molecular weight prepolymers, but the overall amount of molecules would have been reduced due to a higher extent of reaction. Such a difference in mixture characteristics plus the thermal sensitivity of these prepolymers and the aggregation mechanism of the step two reaction suggest that for the 5 min group, a large number of bigger precursor particles aggregated to form hGP7. For the 15 min group, the number of precursor particles available for aggregation was lower and the size of those precursor particles was also much smaller. This resulted in the

morphology observed for hGP9. To support our SEM observations, TEM images of these samples were also collected. The results qualitatively showed that hGP7 was drastically larger than hGP8 and hGP9. Furthermore, comparison of the electron density implies that hGP7 was composed of more precursor polymers than hGP8 and hGP9.

The ζ potentials of these particles were also assessed and summarized in Table 1. Because of the presence of amine groups in the polymer structure (Scheme 3), these particles demonstrated a cationic nature. A higher ζ potential was observed for particles prepared from the 15 min group (i.e., 66 mV for hGP9) than those from the 5 min group (i.e., 56 mV for hGP7). Such a difference likely originates from the extent of reaction, and hence the amount of JT403 that was incorporated into the particles.

The reaction time of the second session incubation was also varied as 30 min, 60 min, 90 min, 120 min, 150 min, and 180 min. The sizes and ζ potential of the obtained particles are very consistent suggesting that particle formation has completed within 30 min in the second session.

2.3.3.3 Reaction temperature

It is well-known that temperature affects the reaction kinetics during step-growth polymerization. To study the influence of reaction temperature on hGP formation, the temperature for the step-one reaction was varied from 55 to 75 °C and the duration was maintained at 10 min. Both DLS and SEM results (Figure 18) revealed that a higher reaction temperature for the first step led to a significant reduction in the size of the final hGPs. The average hydrodynamic diameters of the particles obtained from 55 °C (hGP1), 65 °C (hGP8), and 75 °C (hGP12) reaction were 1000, 600, and 230 nm, with corresponding ζ potentials of 50, 61, and 51 mV, respectively (Table 1). The reaction

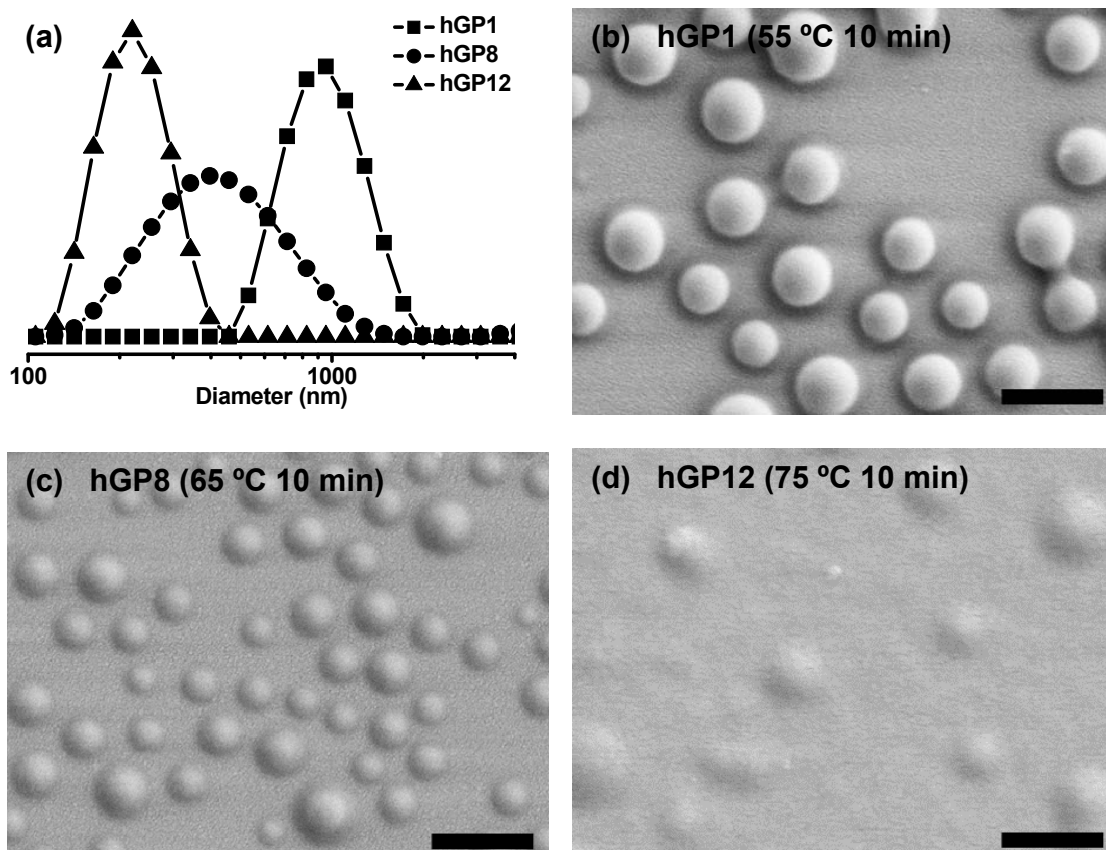


Figure 18 Effects of step one reaction temperature on hGP formation. (a) Representative intensity-averaged hydrodynamic size distribution of hGPs determined by DLS. (b–d) Scanning electron micrographs of hGPs. Scale bar is 1 μm .

temperature also had a direct influence on particle morphology (Figure 18b-d), with particles formed at low temperature showing much more distinct three-dimensionality than those achieved at higher reaction temperatures. The observed effect of reaction temperature on particle size can be attributed to the effect of temperature on reaction rate. At a higher temperature, the step-growth polymerization proceeds faster, leading to higher molecular weight polymers than those produced at a lower temperature for the same duration. Following a mechanism similar to that in the reaction time study described earlier, higher molecular weight polymers formed smaller intermediate precursor nanoparticles, which further aggregated into smaller final hGPs that displayed a severely flattened morphology as revealed by the SEM image of the dried samples (Figure 18d).

2.3.3.4 Monomer concentration

The effect of the initial monomer concentration (JT403 + BDE) to hydrogel particles (hGP) formation was investigated by varying the reactant concentration from 5 to 15 wt% during the first session, while keeping the reaction temperature set at 65 °C and reaction time at 15 min. DLS data (Table 1 and Figure 19) showed that the average diameter of final hGPs decreased from 790 nm (hGP5) produced from 5 wt% monomer, to 510 nm (hGP6) from 10 wt% monomer, to 410 nm (hGP9) produced from 15 wt% monomer content, and the corresponding ζ potential increased from 47 mV to 49 mV and 66 mV, respectively. This inverse correlation between the particle size and the monomer concentration was presumably a result of how the monomer concentration affects the polymer molecular weight. It is expected that the epoxy–amine reaction proceeded faster as the monomer concentration increased, thus leading to a higher molecular weight

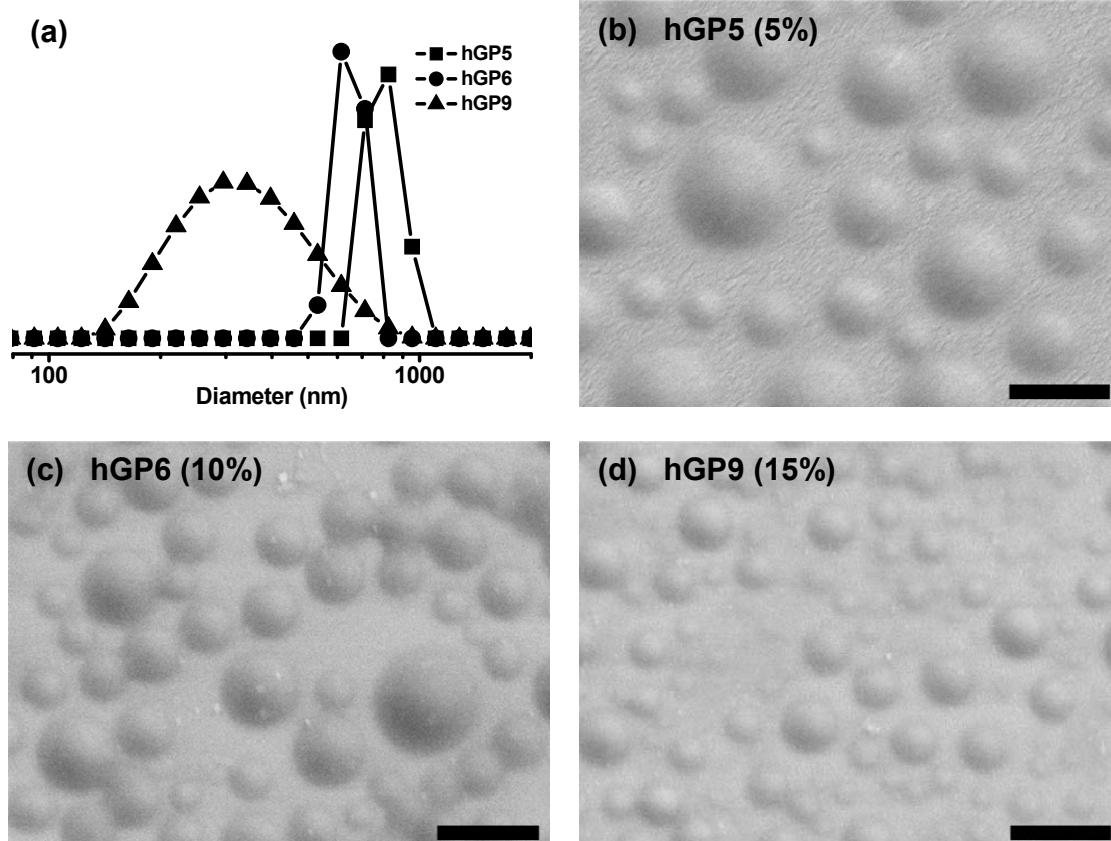


Figure 19 Effects of step one reactant concentration on hGP formation. (a) Representative intensity-averaged hydrodynamic size distribution of hGPs determined by DLS. (b–d) Scanning electron micrographs of hGPs. Scale bar is 1 μm .

polymer formation within the same amount of reaction time. Considering the correlation between the polymer molecular weight and the final particle size, the observation of smaller particle formation at a higher monomer concentration was rationally accounted for. The influence of monomer concentration on the particle morphology was less drastic as compared to that exerted by reaction time and temperature. The SEM micrographs showed that hGP5, hGP6 and hGP9 particles all appeared to be round but with relatively low three-dimensionality (Figure 19b–d). The lack of strong dimensionality in the 5 wt% sample can be explained by the low initial reactant concentration leading to the production of a smaller quantity of low molecular weight prepolymers (i.e., large precursor particles). The phase separation of low molecular weight prepolymers at low degree resulted in the low crosslink density and low dimensionality of 5 wt% particles. On the contrary, with high crosslink density, the 15 wt% particles are highly hydrophilic considering the high density of cationic amino groups (at ζ potential of 66 mV). The high hydrophilicity of the network caused the loss of dimensionality of the particles on the hydrophilic silica surface during the air-drying process.

The concentration of prepolymer in the second session was varied as 0.1 wt%, 0.25 wt%, 0.5 wt%, and 1.0 wt% while the concentration of the first session was kept 15 wt%. The hydrodynamic sizes of the crude products before purification are 71 nm, 82 nm, 108 nm, and 130 nm as concentration increased from 0.1 wt%, 0.25 wt%, 0.5 wt%, to 1.0 wt%. The purification of suspensions with such a small size around 70 nm is not straightforward. The particles could not be collected even at the highest speed of 21 kRCF using the available centrifuge. Therefore the sizes and size distributions of the crude products are presented (Figure 20) for understanding the effects of the reactants

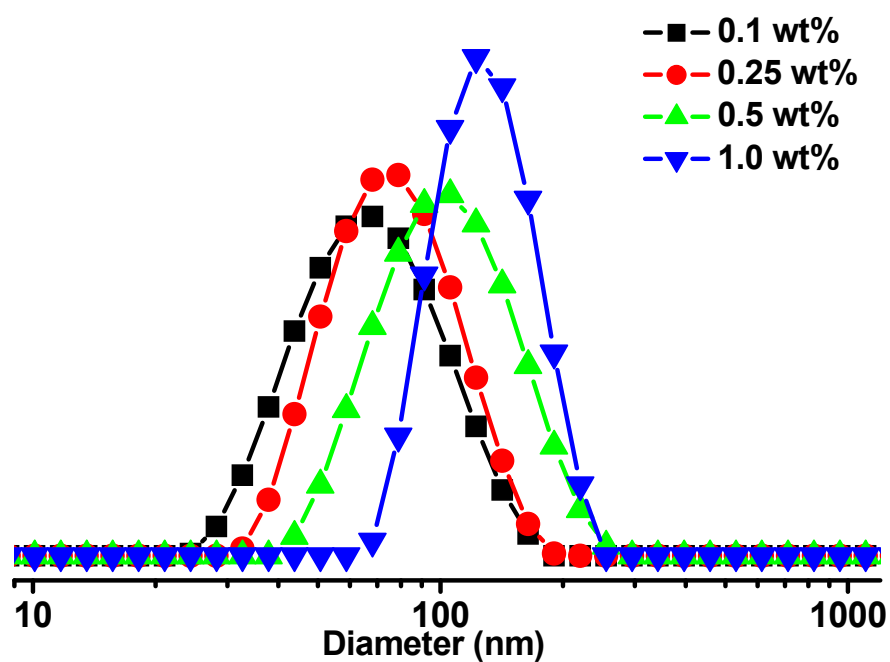


Figure 20 Effects of reactant concentration in second session on hGP formation. Representative intensity-averaged hydrodynamic size distribution of hGPs from concentration ranged from 0.1 wt% to 1.0 wt% by zetasizer.

concentration.

The observed increase in size as a function of concentration of the prepolymer in the second session can be explained based on possibility of coacervates in aggregation. In this study, the same prepolymer obtained from the first session was used. So for all concentrations in the second session, the extent of phase separation of the prepolymers are comparable because all the influential parameters are the consistent including polymer composition, molecular weight, and solution temperature. The prepolymers should form coacervates of same size at a very small scale. The coacervates then aggregated into precursor nanoparticles and continued coupling reaction to form crosslinked gel network. Therefore the sizes of the final particles are dependent on the quantity of coacervates in forming each particle. At high concentration as 1.0 wt%, there was greater possibility of coacervates meet with each other and aggregate to form larger particles, comparing to the low concentration such as 0.1 wt%.

2.3.3.5 Monomer composition

The well-established system provided various particles with properties tunable in a wide range. For example, the ζ potential of hydrogel particles could be adjusted between 48 mV and 71 mV. However, for biomedical application, lower charged density is demanded to improve the biocompatibility of drug carrier. Therefore we introduced a third monomer into this system. In order to keep the integrity of the backbone, a new monomer named Jeffamine ED 900 (JED900) was selected. Similar to JT403, JED900 is also a commercially available monomer with PO structure in the backbone and amino groups at the end of molecular chains. But JED900 contains a higher portion of EO units than JT403. Unlike the trifunctional JT403, JED900 is a linear molecule with two

primary amino groups (Figure 21). The particles were prepared while the monomer stoichiometric ratio of amino hydrogen to epoxy was kept constant as 2:1, and the ratio of amino hydrogen between JT403 and JED900 varied as 6:0 and 6:3. The new particles of 6:3 were named TED hydrogel particles. Both compositions provided well-defined nanogels dispersed in a stable homogenous colloidal suspension without any aggregation. After purification, there was a clear trend of decrease in turbidity of nanogels suspensions at the same concentration when JED900 was used as one of the monomers (Figure 22). It was speculated that the apparent hydrophobicity of nanogels decreases with the incorporation of JED900. The average hydrodynamic diameters of the T and TED particles were 270 nm and 240 nm, respectively, with similar size distributions. The TEM images of dehydrated samples showed spherical morphologies of particles. The particles shrink in volume during the dehydration process [74]. The zeta potentials of TED particles decreased from 65 mV of T particles to 45 mV when the amino hydrogen ratios between JT403 and JED 900 decreased from 6:0 to 6:3. Specifically, the JT403 is a tri-functional monomer at the size of 440 g/mol with 3 amine groups while the JED900 is a linear bi-functional monomer at the size of 900 g/mol with 2 amine groups. Theoretical estimation indicates that in a stretched molecule, the average distance between the neighboring amine groups in a JED900 molecule is 1.8 times of that in a JT403 monomer. Therefore, a reduction in the density of amine groups in the network of particles containing JED 900 is expected, which also explains a lower ζ potential of TED as compared to T particles of similar size

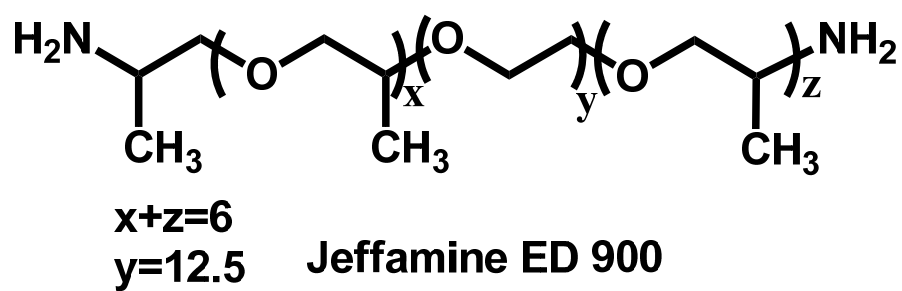
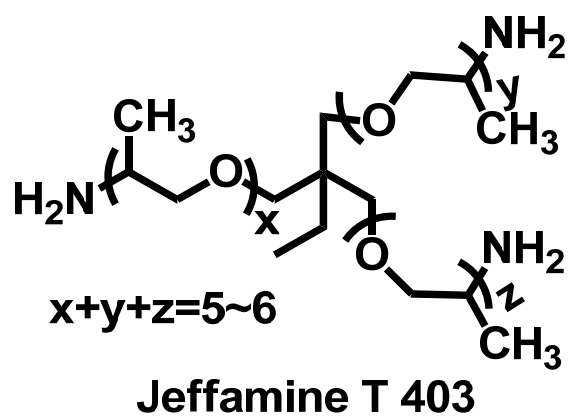


Figure 21 Chemical structures of Jeffamine T 403 and Jeffamine ED 900.

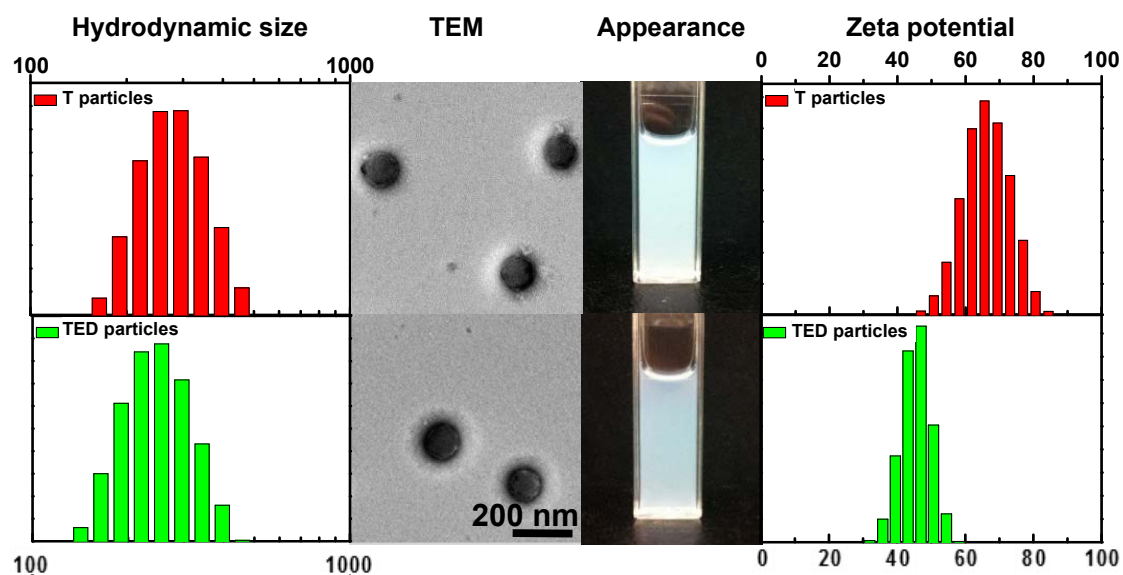


Figure 22 Distribution of size and charge density of hydrogel particles determined by zetasizer in water. Sizes of dehydrated particle were investigated using TEM. Optical appearances were obtained from particle suspensions.

2.3.3.6 Further discussions on particle preparation-property relationships

The particle properties including size and charge density are tunable via monomer ratio, monomer concentration, reaction time and reaction temperature. To achieve the desirable properties, it is more straightforward to adjust reaction time and reaction temperature than to change monomer concentration and monomer ratios.

The influence of reaction time and reaction temperature toward sizes were summarized in Figure 23. As previously discussed in details, the sizes decreased as reaction time increased and reaction temperature increased due to the increase of χ and resulting increase of degree of phase separation. The effects toward ζ potentials were also summarized (Figure 24). According to the comparison between 55 °C and 65 °C, the charge density increased as temperature increased. The increase of reaction time also resulted in increase of ζ potential.

In addition to hydrogel particle size, charge, and morphology, it is important to know how the synthesis parameters influence the temperature dependent swelling characteristic of these hGPs. Our study showed that bisepoxide-Jeffamine derived hydrogel particles exhibited thermoresponsiveness analogous to that found with Pluronic polymers [67]. The thermoresponsiveness of swelling/de-swelling in size is likely originated from the hydrophobic-hydrophilic structure of the hydrogel particles, exemplified by the poly(propylene oxide)-rich domains linked by hydrophilic amine and diol groups (Scheme 3). As summarized in Table 1, for all the synthetic conditions resulting in the formation of hGPs, those particles were thermal responsive by showing volume reduction at higher temperatures. A wide range of swelling ratios, defined as particle volume at 0 °C to volume at 45 °C, was particles were thermal responsive by

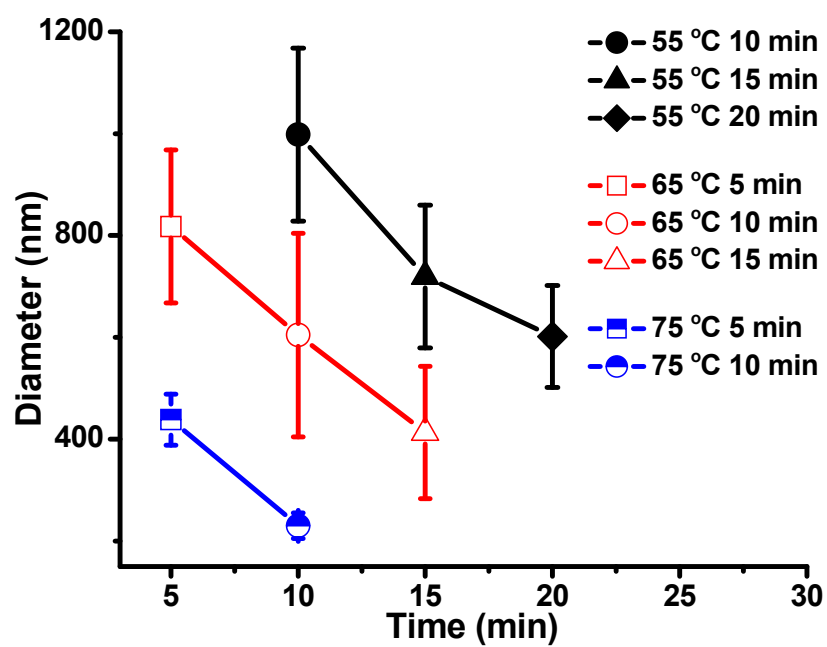


Figure 23 Summary of particle sizes as a function of reaction time and temperature.

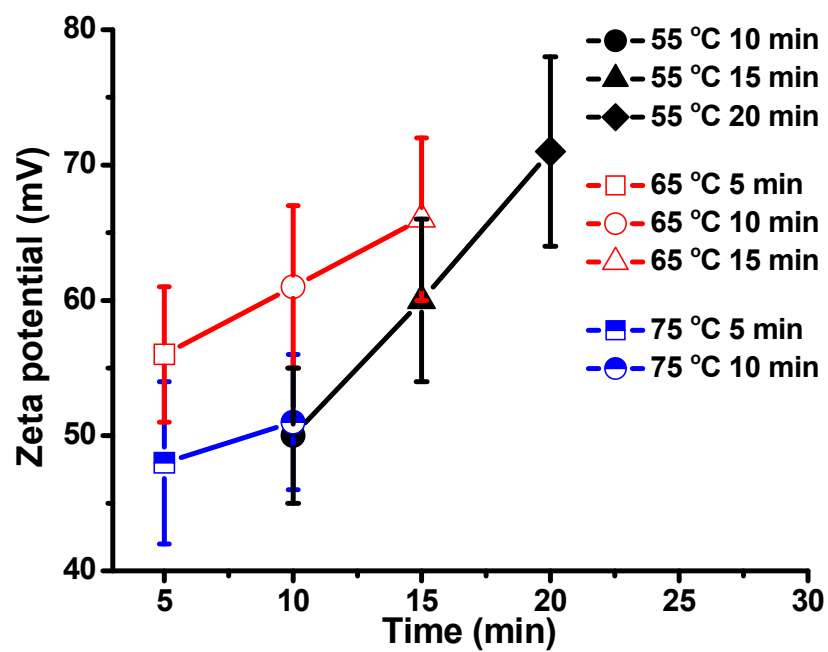


Figure 24 Summary of particle ζ potentials as a function of reaction time and temperature.

showing volume reduction at higher temperatures. A wide range of swelling ratios, defined as particle volume at 0 °C to volume at 45 °C, was observed for these hGPs, spanning from 2.7 to 18.5. Such a diverse range implies that these particles differ significantly in the epoxy-amine network density, which was presumably influenced by the different synthesis parameters.

The thermoresponsiveness of hGP3, hGP9, and hGP12 was further investigated in detail by monitoring their swelling ratios as a function of temperature. These three samples were selected as each one corresponded to the condition closest to macroscopic gelation when different reaction temperatures were used during step one of the particle preparation protocol. In other words, these samples represented the most structurally established group in their respective pool. As shown in Figure 25, all hGPs demonstrated a continuous volume phase transition from swollen to de-swollen states as the solution temperature increased. However, the shape of the swelling-deswelling curves was drastically different among the hGPs. Within the temperature range studied (0-45 °C), hGP3 and hGP9 underwent a broad volume phase transition over the entire temperature range (Figure 25a-b), while hGP12 exhibited transition in a narrower range of 30 to 45 °C (Figure 25c). Additionally, there was a shift in the temperature that corresponded to the sharpest transition for each hGP. For hGP3, the steep transition was observed for 0-15 °C; for hGP9, it occurred around 25 °C; and for hGP12, the sharp change came around 40 °C. The higher transition temperature of hGP12 is likely due to its relatively high hydrophilicity. Since hGP12 was formed under the highest reaction temperature, its constituent polymer naturally corresponded to the product generated from the highest extent of reaction between epoxy and amine. Thus, with more diepoxide reacted, a higher

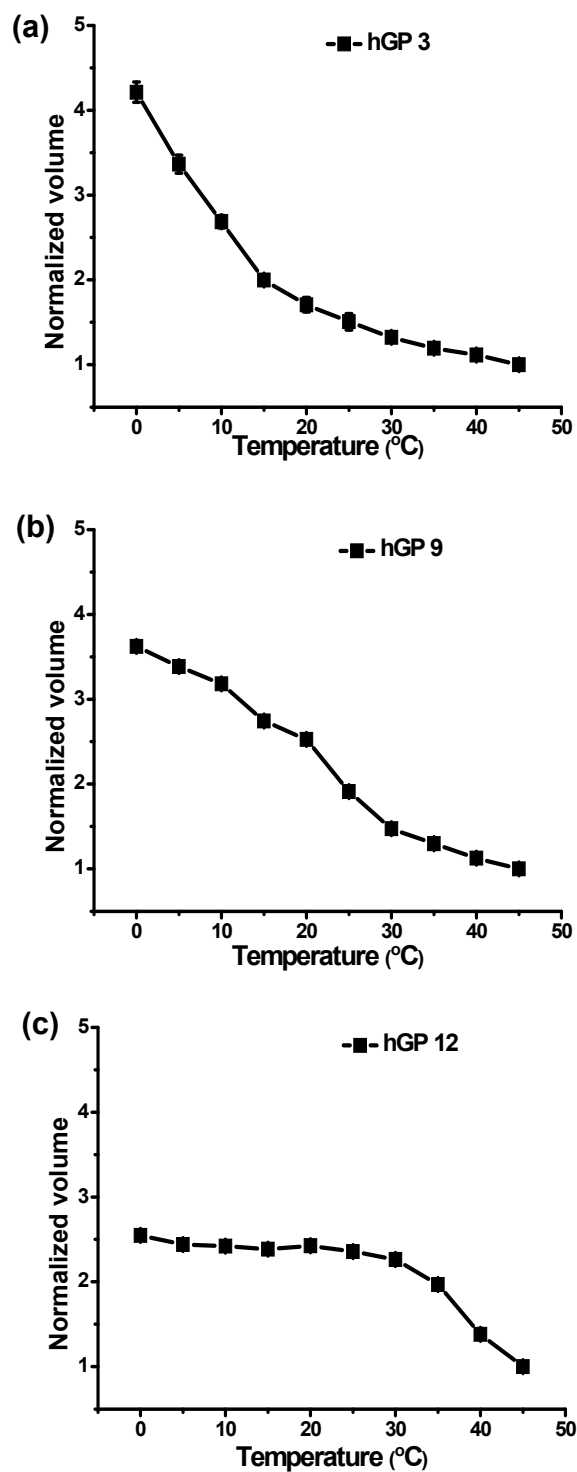


Figure 25 The normalized volumetric swelling vs. temperature for (a) hGP3 (55 °C, 20 min), (b) hGP9 (65 °C, 15 min), and (c) hGP12 (75 °C, 10 min).

amount of the hydrophilic diol groups were formed and incorporated in the network, making the final hydrogel particle more hydrophilic. Previous studies have shown that, for polymers exhibiting lower critical solution temperature behavior, an increase in polymer hydrophilicity will generally raise its phase transition temperature [34, 75-77].

2.3.4 Cytocompatibility of micro/nano-gels and application for drug delivery

2.3.4.1 Cytocompatibility of micro/nano-gels

A very important aspect of developing these hydrogel particles to be of biomedical use is to ensure good biocompatibility, especially considering their cationic nature. As a first-line evaluation, we examined cytocompatibility of our hydrogel particles using *in vitro* assays. Macrophages were employed for the *in vitro* study because of their high sensitivity to the toxicity of various macromolecules and nanoparticles [78, 79], as well as their critical roles as physiological scavengers of foreign materials [80]. A highly water-soluble tetrazolium salt based WST-1 assay was used to study the cytotoxicity of various gel particles prepared under the aforementioned conditions. The intensity of measured light absorption from WST-1 assay directly correlates to the metabolic activities or the viability of cells. In order to establish the range of testing concentrations, particles made from the typical reaction condition reported in our previous study (e.g. hGP9, 65 °C, 15 wt%, 15 min) with well-characterized responsive behaviors were used. Viability of cells was then analyzed after exposure to various concentrations of hGP9. The histogram in Figure 26 demonstrates that cell viability decreases with the increase of hydrogel particle concentrations. Up to 0.5 µg/mL of particle exposure, no evident cytotoxicity was observed. However, significant cell death was induced at higher concentrations, where 10 and 50 µg/mL of particles resulted in less

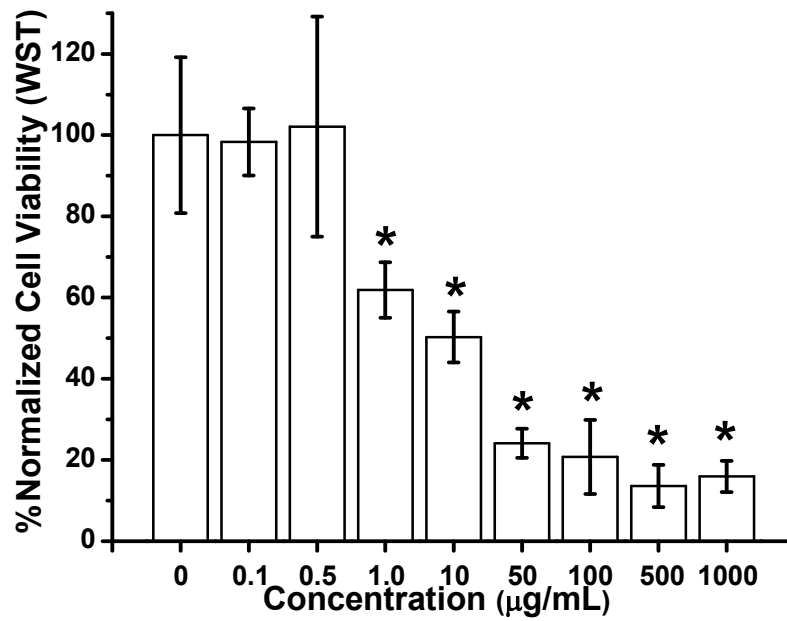


Figure 26 The RAW264.7 macrophage viability after 4 h treatment with typical hydrogel particles hGP9 (65 °C, 15 min) at different concentrations. Comparison was made with the non-treated control group and statistical significance was observed at * $p < 0.05$.

than 50% and 25% cell survival, respectively. These three concentrations were thus selected for subsequent cytotoxicity studies of hydrogel particles prepared under various reaction conditions.

As seen from Table 1, different reaction conditions greatly affected the resultant particle surface charges, with values spanning from 47 mV to 71 mV. In order to understand the influence of such high surface charges on hydrogel particle cytotoxicity, we have selected two pairs of hGPs that are comparable in sizes but differ in ζ potentials for cell viability assays. Specifically, particles with hydrodynamic diameters around 600 nm (hGP3 and hGP8) and around 400 nm (hGP9 and hGP11) were introduced into cell culture medium. As a negative control, a cationic polymer, PEI, with known cytotoxicity [81, 82] and chemically sharing some similarity with our hydrogel particles, was also used in parallel for cell treatment. The normalized cell viability (Figure 27a) showed that all the hydrogel particles tested and the PEI control disturbed cell survival in a concentration dependent manner. Such a concentration dependent cytotoxic effect can be attributed to that the amount of charged subjects imposed on individual cells increases as the overall treatment dosage increases, a phenomenon which was also reported for other cationic polymers [78, 82]. The cationic polymers interact with the cell membrane inducing the formation of transient holes and resulting in the malfunction in living cells. At the lowest working concentration tested (0.5 $\mu\text{g/mL}$), a slight decrease in cell viability was noted only for the hGP3 group, though not statistically lower than the untreated control. When the concentration was increased to 10 $\mu\text{g/mL}$, a statistically significant decrease in cell viability as compared to the untreated control was observed for all the hydrogel particle groups except hGP11. However, the extent of cytotoxic effects varied,

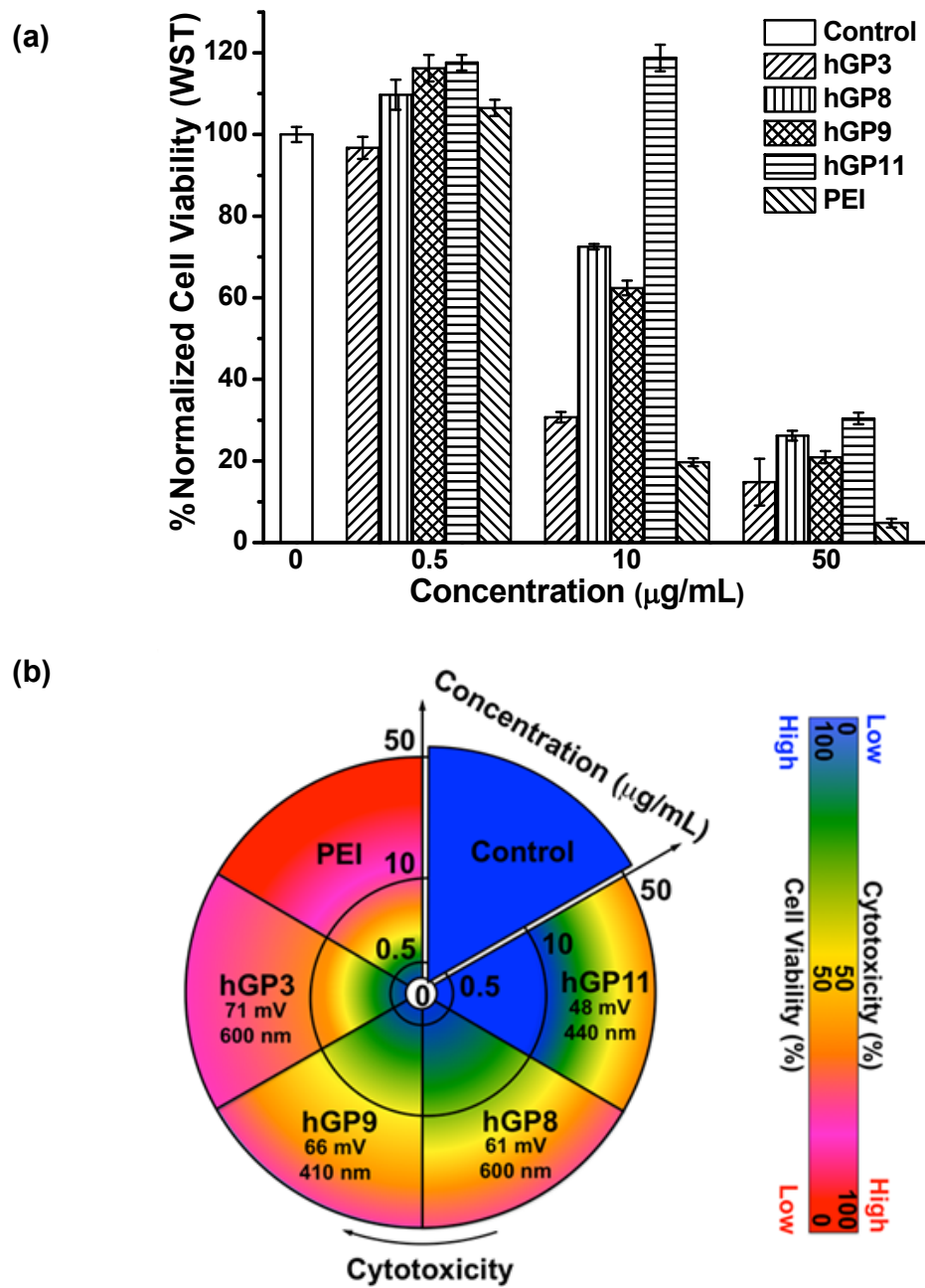


Figure 27 The cytotoxicity of hGP3 (55 °C, 20 min), hGP8 (65 °C, 10 min), hGP9 (65 °C, 15 min), hGP11 (75 °C, 5 min), and PEI at different concentrations to RAW264.7 macrophages for 4 h exposure. (a) Cell viability; (b) A full spectrum presentation for the cytotoxicity of indicated polycations.

with hGP3 showing significantly lower ($p < 0.01$) cell viability (~30%) at this concentration when compared with hGP8 and hGP9, both of which had cell viability above 50%. When the concentration was further increased to 50 $\mu\text{g/mL}$, all hydrogel particle groups triggered substantial cell death ($> 50\%$) suggesting the cytotoxicity is dosage dependent. Though in comparison to the PEI control, hGP8, 9, and 11 particles were significantly ($p < 0.05$) less cytotoxic at this concentration.

Overall, the viability results suggested that cytotoxicity of these particles along with PEI ranked in the following order: hGP11 $<$ hGP8 $\approx$ hGP9 $<$ hGP3 $<$ PEI. A full spectrum has also been schematically demonstrated in Figure 27b to illustrate the cytotoxicity of these tested materials. The increase of material cytotoxicity or decrease of cell viability was represented by the change of color from blue to red. This color-coding system was developed to facilitate the following discussions on the correlation between surface charges of the hydrogel particles and their cytotoxicity.

It is clear that for both pairs of particle diameters (600 nm and 400 nm), strong dependency of cytotoxicity on particle surface charge was observed. The 71 mV ζ potential exhibited by hGP3 made it less cytocompatible even at the lowest testing concentration of 0.5 $\mu\text{g/mL}$. While for hGP8, carrying a ζ potential of 61 mV made it more cytocompatible than hGP3, suggesting a reduction in the particle surface charge was more favorable for cell viability. A similar trend was noted for the pair of hGP9 and hGP11, with the much less positively charged hGP11 (48 mV) showing a higher percentage of cell survival than hGP9 (66 mV) under both testing concentrations of 10 and 50 $\mu\text{g/mL}$. Our findings of the inverse relationship between positive surface charge density and biocompatibility of cationic particles are consistent with a number of

previous studies highlighting the important effect of biomaterial surface charge on its biocompatibility. For example, Fischer et al. investigated the cytotoxicity of a variety of water-soluble polycationic macromolecules *in vitro* [82]. They concluded that the cationic charge density of the polymers is a key parameter in inducing cell damages, where high charge densities caused higher cytotoxic effects than those with lower cationic charge densities. Similar observations have been reported on cationic dendrimers [83], as well as positively charged polystyrene [79], gold nanoparticles [84], and quantum dots [85]. Recent mechanistic studies have advanced our understanding of the principles underlying the great cytotoxicity associated with highly charged cationic polymers or micro/nanoparticles. It is hypothesized that the cytotoxic effect is largely attributed to the strong electrostatic interactions between these cationic materials and the negatively charged cell membrane, which could result in significant disruption of the membrane integrity and concomitant cytotoxicity [86-88].

Parallel to the quantitative analysis of cell viability through the metabolic WST assay, morphological features of cells were also examined qualitatively in our study to gain further insight into the biocompatibility of hydrogel particles. As shown in Figure 28, distinct changes in macrophage morphology were observed in a treatment-dependent and concentration-dependent manner. At the concentration of 0.5 $\mu\text{g/mL}$, cells in all the treatment groups have grown into a near-confluent layer with morphology similar to those in the non-treated control group (Figure 29). At a higher working concentration of 10 $\mu\text{g/mL}$, cells in most of the treated groups, except hGP11, started to lose their integrity and the number of intact cells decreased after the treatments. A greater extent of cell fragmentation, higher amount of cell debris, and fewer adherent cells were observed

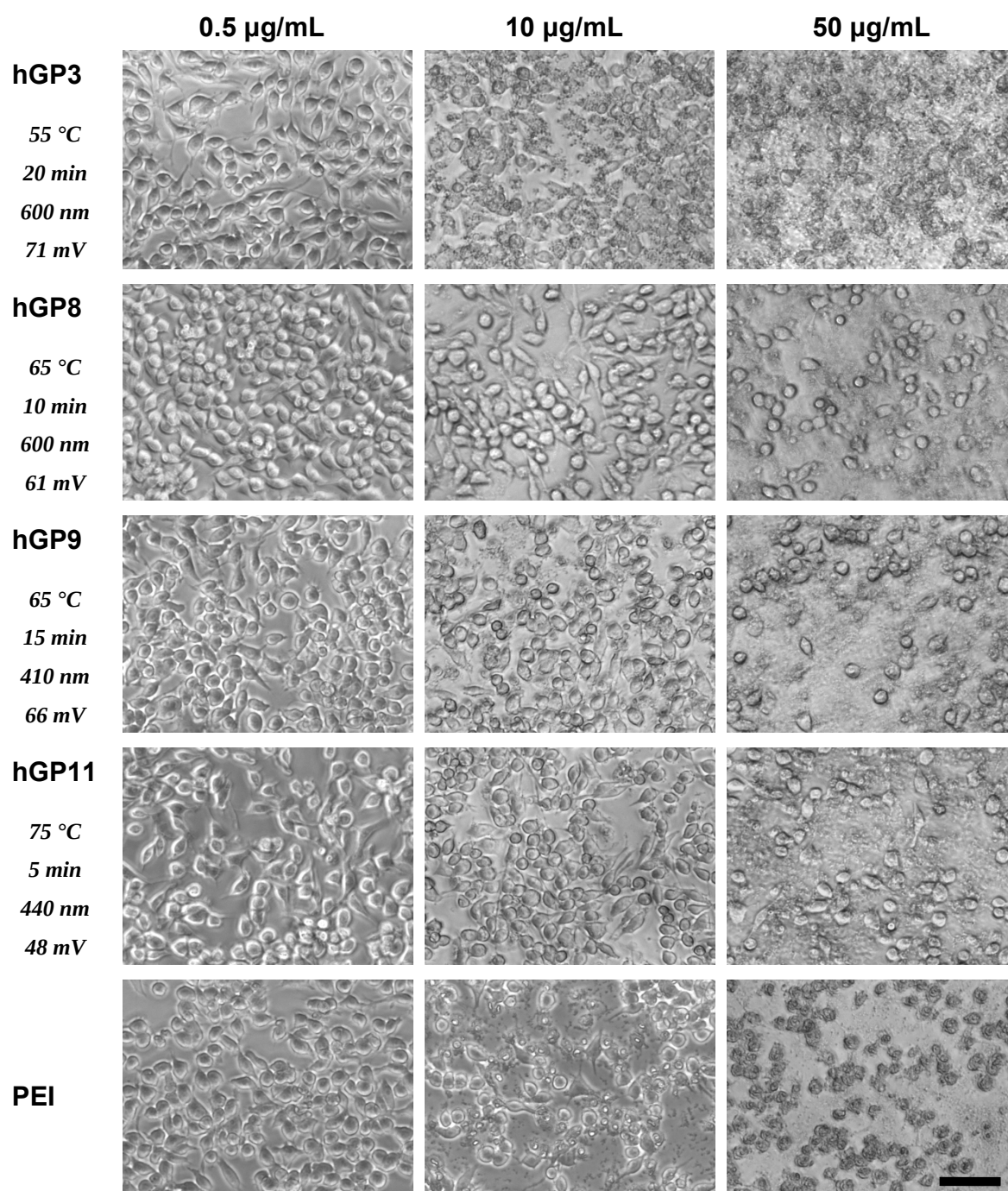


Figure 28 Representative phase contrast micrographs of the RAW264.7 macrophage cells after incubation with various concentrations of hGPs or PEI. Scale bar is 50 μm .

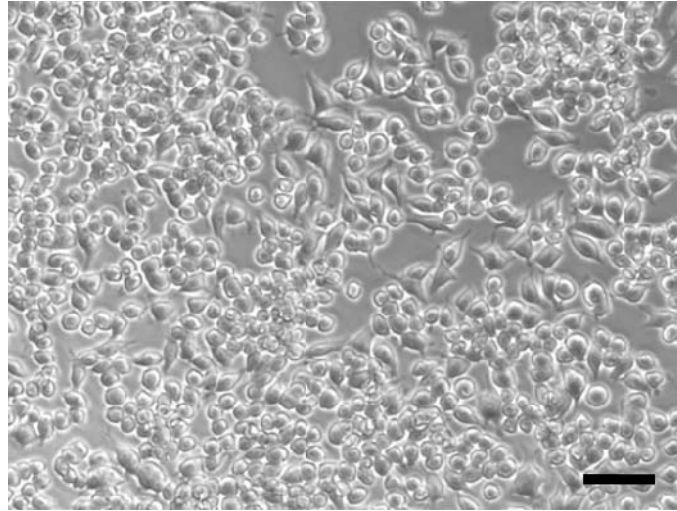


Figure 29 Morphology of the untreated RAW264.7 macrophages. Scale bar is 50 μm .

when the concentration reached 50 $\mu\text{g/mL}$. These microscopic observations on cell numbers and morphologies suggested that hGP3 exerted the most damage to macrophages, while hGP11 was the least cytotoxic. These results were in good agreement with the WST assay.

Considering the suggested role of strong interactions between cationic materials and cell membranes in inducing cell death, we used SEM to closely study macrophage cell membrane integrity after incubation with cationic hGPs. Results obtained from the 10 $\mu\text{g/mL}$ treatment were presented in Figure 30. Under high magnification, the non-treated macrophage control displayed regular cell morphology with a rough surface featuring a great amount of long fibrous microvilli. In contrast, cells receiving treatments showed clear alterations in surface morphology. First of all, cells appeared to be osmotically swollen and cell membrane surface became less rugged, indicating that cells had lost their osmotic balance and the membrane permeability had been substantially increased [89]. Secondly, a significant loss of microvilli structures from cell membrane surface was observed. Lastly, cytoplasmic blebbing was also evident for cells treated with hGP3 and hGP9, a phenomenon that was more pronounced in the PEI control group due to its high cytotoxicity [90, 91]. In general, these observations indicated that, similar to the reference polymer PEI, the cationic nature of our hydrogel particles contributed to the adverse effects on cell membrane permeability and structure. The higher the charge it carries, the more severe the damage it induces, hence the greater cytotoxicity. This trend is consistent with the conclusion drawn from our WST assay. The qualitative morphological alteration analysis provides an alternative calibre to gauge the cytotoxicity of materials [91, 92]. Furthermore, the formation of blebs on cell surfaces suggests that

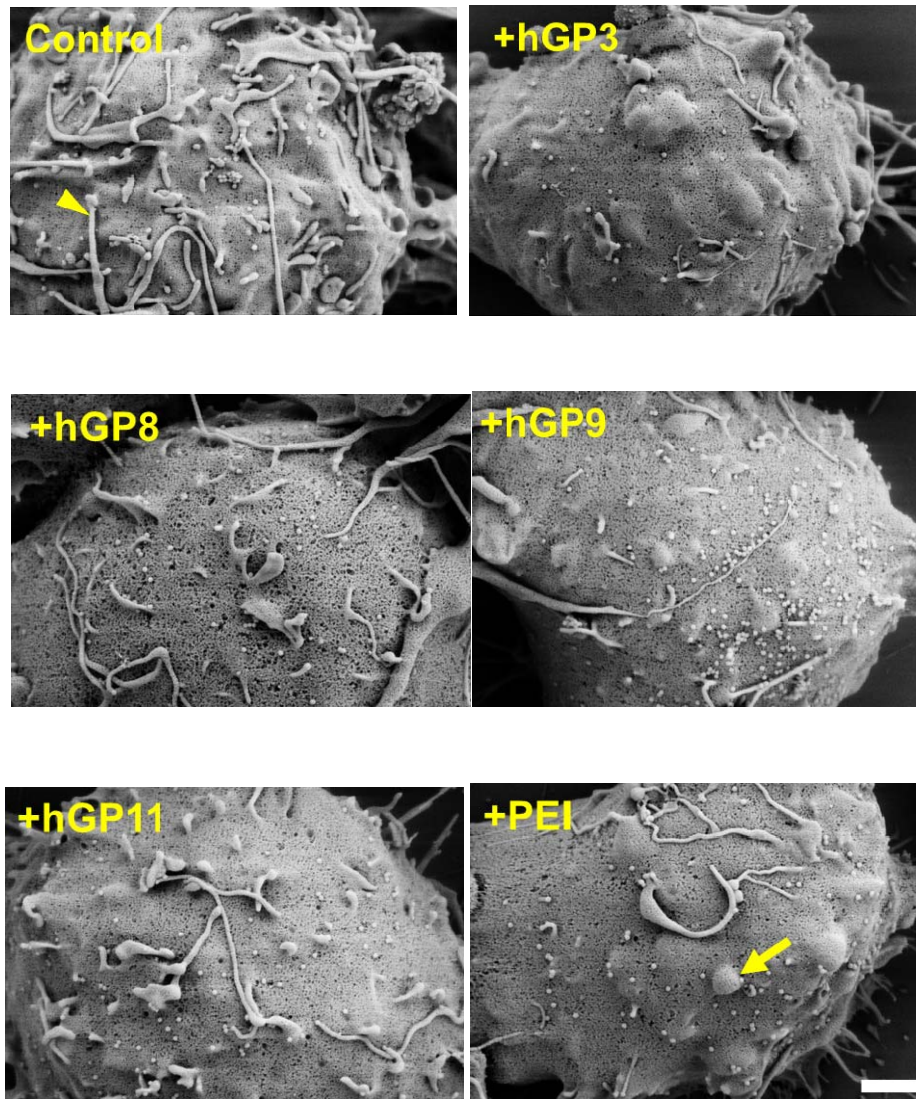


Figure 30 SEM micrographs showing the effects of various types of hGPs (10 $\mu\text{g/mL}$) on macrophage cell morphology and membrane integrity. Untreated control and PEI treated cells are included for comparison. Arrow head (control) indicates microvilli structure and arrow (PEI) indicates cytoplasmic blebbing. Scale bar is 1 μm .

cell death in the presence of these hGPs is potentially following an apoptotic mechanism [93]. These morphological findings can guide future in-depth studies to shed light on the molecular mechanisms of cell death induced by this new family of cationic hydrogel particles and in turn allow further improvement of their biocompatibility for biomedical considerations.

2.3.4.2 Application of micro/nano-gels for drug delivery

The micro/nano-gels particles prepared in this system contains hydrophobic PPO domains which is perfectly suitable for loading and delivering the hydrophobic biomolecules because of hydrophobic interaction. To examine the capability of this nanogel system to uptake and release therapeutic chemicals, we used a hydrophobic dye, Nile red (NR), as a guest molecule for stimuli-responsive release study [94]. Specifically, NR release can be probed by fluorescence spectroscopy because of the dramatic decrease in the emission intensity from NR in a hydrophilic environment such as water as compared to that in a hydrophobic condition.

It was found the NR was successfully loaded to the particles. As shown in Figure 31, the blank T nanogels exhibited no fluorescent signal under the excitation at 550 nm. On the contrary, the NR containing T nanogels fluoresced at the wavelength of 633 nm with a peak value of 334 a.u..

Then the responsiveness of particles toward temperature was investigated using NR-loaded T particles. At 0 °C, the dye in the particles showed a very low fluorescent intensity (Figure 32a) because the swelling of the hydrogel particles resulted in a relatively more hydrophilic local environment around the NR. As temperature was raised from 0 °C to 40 °C, the peak value of the emission increased dramatically (Figure 32b). It

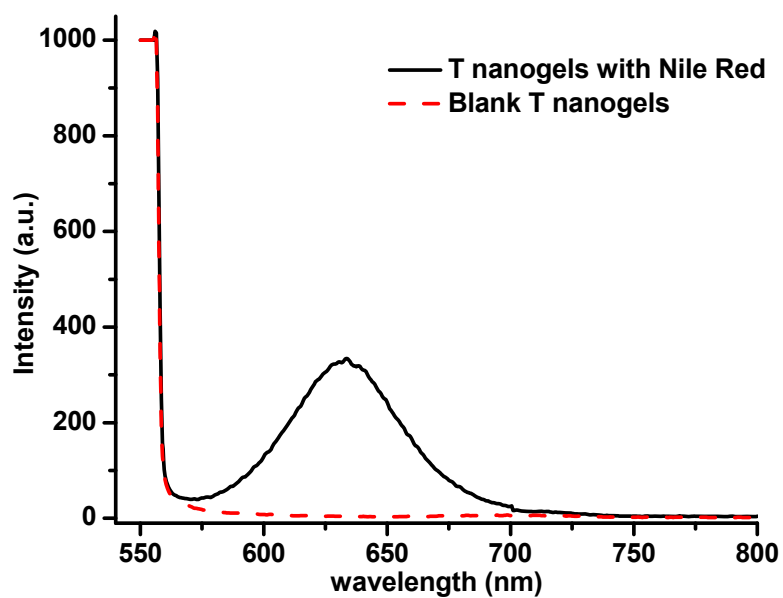


Figure 31 Fluorescent emission spectra of Nile Red loaded T nanogels and blank T nanogels with excitation at 550 nm at room temperature. The particle concentrations are 1 mg/mL.

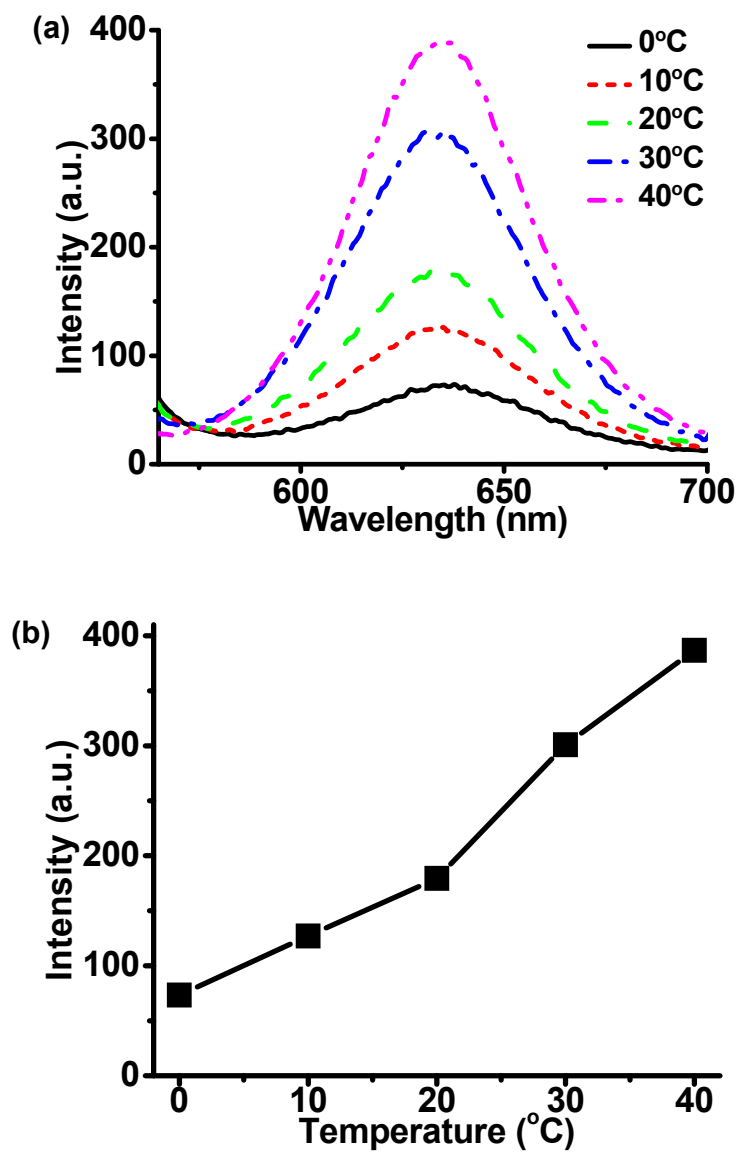


Figure 32 Fluorescent emission spectra (a) and emission peak intensities (b) of Nile Red loaded T nanogels at different temperatures with excitation at 550 nm. The particle concentration is 1 mg/mL.

is noteworthy that the sharpest transition took place between 20 °C and 30 °C, consistent with the observation in the size change in response to thermal stimulus based on the DLS studies.

The pH responsiveness was also studied with NR-loaded T particles. The pH of the aqueous particle suspension was adjusted from 7 to 13 gradually in order to monitor the variance of emission intensity. It was observed in Figure 33a that the peak intensity of the emission increased as a function of solution pH. Then the pH was reduced via addition of acid into the solution with pH at 13. It is interesting that the fluorescent intensity maintained until the pH decreased to 7. The interaction between the hydrophobic dye and hydrophobic domains of particles could account for the delayed response toward pH in the basic environment. When pH was reduced to 5 and lower, the emission intensity decreased drastically due to the swelling of particles.

Compared with pH and temperature, oxidation-triggered release is less investigated in literature, yet it is highly relevant in a biomedical context. Upregulation of potent oxidants has been associated with many pathological diseases [95]. Thus, it will be advantageous to use oxidation as a trigger to achieve controlled drug release. When the Nile red containing particle suspension was treated with 1 vol% hydrogen peroxide (Figure 11), the encapsulated NR was released into water as the particles being rapidly degraded by H₂O₂. Since water is a non-solvent for NR, the released NR precipitated to the bottom of the cuvette accompanied by a gradual decrease of its emission intensity as a function of time (Figure 34a-b). The results suggest that our particle is applicable for oxidant triggered release of hydrophobic drugs.

The loading of NR was carried out via a classical infiltration method to the T and

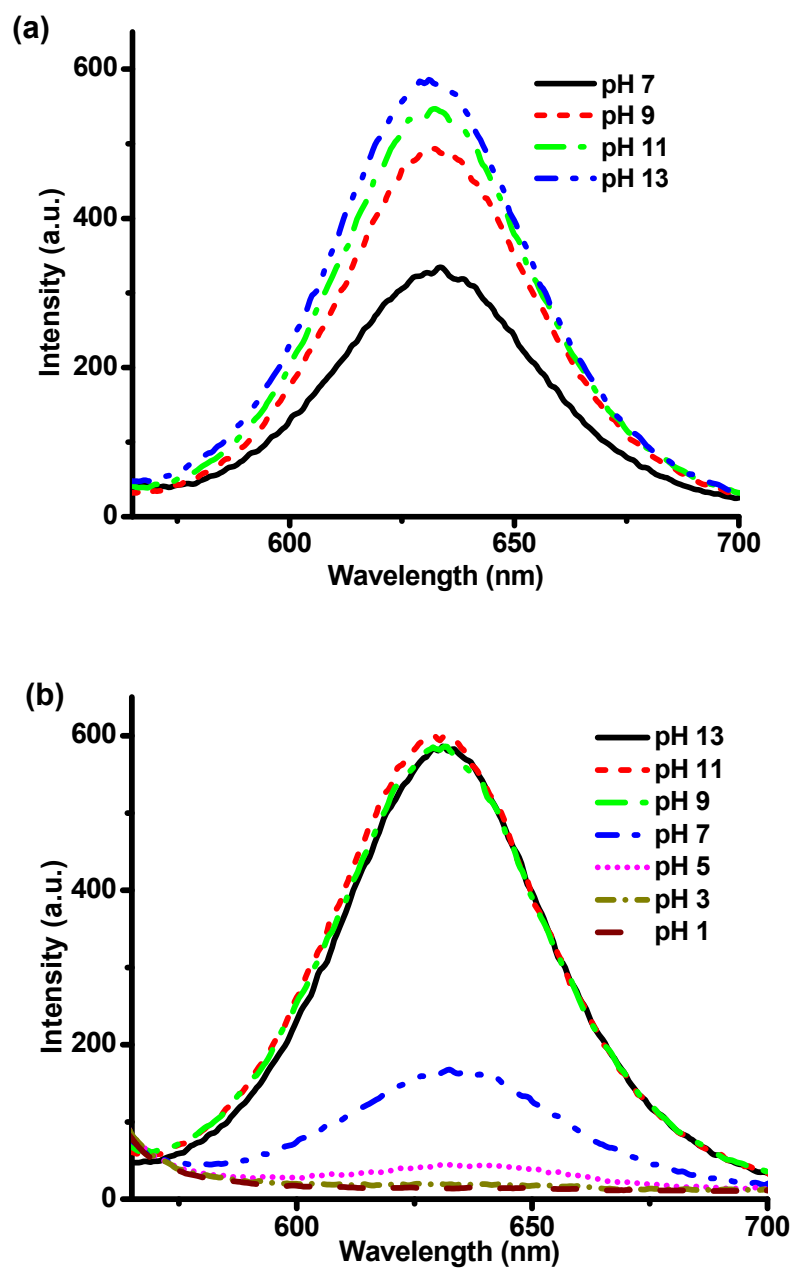


Figure 33 Fluorescent emission spectra of Nile Red loaded T nanogels at different pH with excitation at 550 nm. The particle concentration is 1 mg/mL. (a) The pH value of solution was initially adjusted from 7 to 13, and then was (b) tuned from 13 to 1.

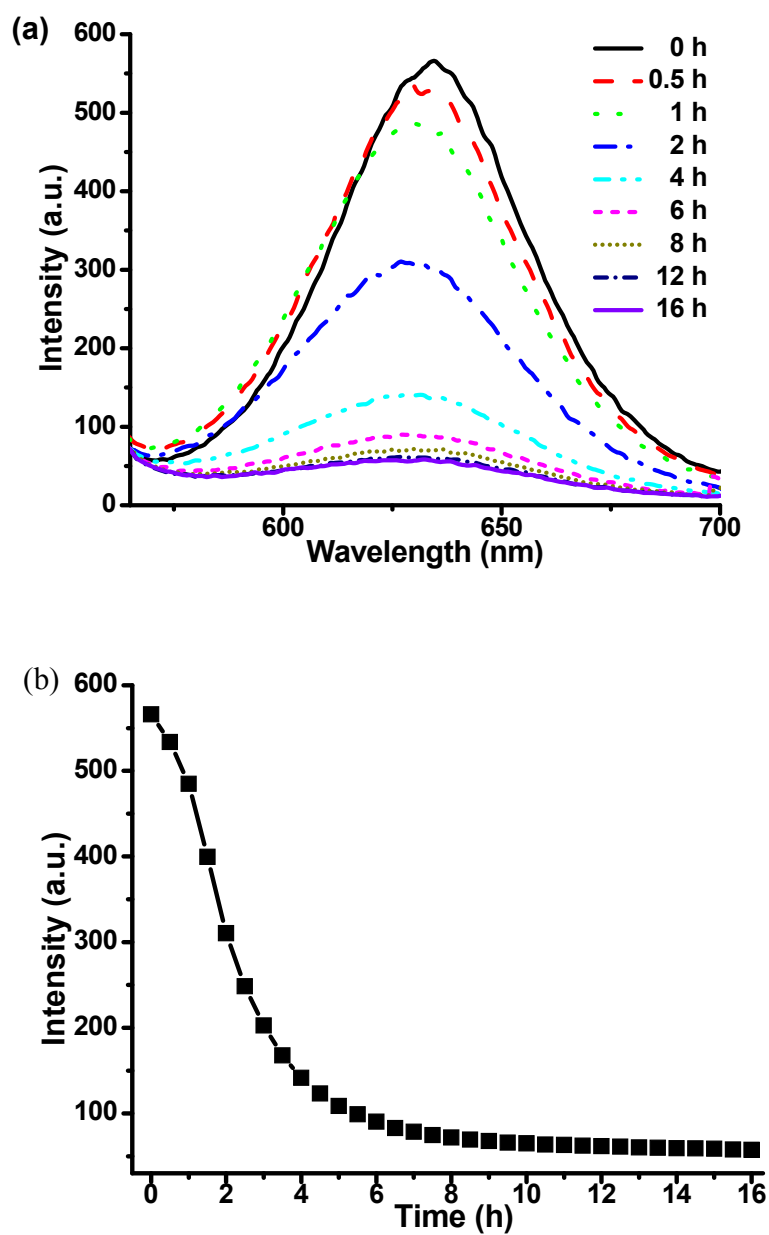


Figure 34 Fluorescent emission spectra (a) and emission peak intensities (b) of Nile Red loaded T nanogels during degradation in presence of 1 wt% hydrogen peroxide with excitation at 550 nm. The particle concentration is 2.5 mg/mL.

TED particles for comparison. It was found that similar to T particles, the NR was successfully loaded to the TED particles. The optical macrographs of NR-loaded T and TED samples exhibited similar appearance in color as shown in inset of Figure 35. But the difference was observed in the fluorescent emission spectra of the two samples. The emission spectrum of NR-loaded TED particles indicated a peak at 637 nm, which was about 4 nm red shift from that of T particles at 633 nm. The fluorescence of Nile red is strongly influenced by the polarity or hydrophobicity of its local environment [94]. Red shifting of NR fluorescent emission peak wavelength occurs in the aqueous media. The 4 nm red shift from T to TED particles suggested a higher hydrophobicity in the local environment of the T particles. This is expected as T and TED particles differ in composition, where the additional PEO domains from JED900 have enhanced the hydrophilicity of the obtained TED particles

2.4 Conclusions

A straightforward and environmentally friendly method was developed to prepare micro/nanogels capable of responding to temperature, pH, and oxidants. Formation of the nanogels is facilitated by secondary aggregation of precursor nanoparticles resulting from thermal sensitive intermediate polymers produced *in situ*. The remarkable volume change of the hydrogel particles in response to temperature and pH, and degradation capability in presence of oxidants offer promising opportunities for fast stimuli-triggered drug delivery. Properties control including size, ζ potential, and swelling behavior of the hydrogel particles can be properly achieved by varying the reaction parameters such as monomer concentration, monomer ratio, reaction time, and reaction temperature. In addition, an inverse relationship between particle sizes and reaction time, temperature, and reactant

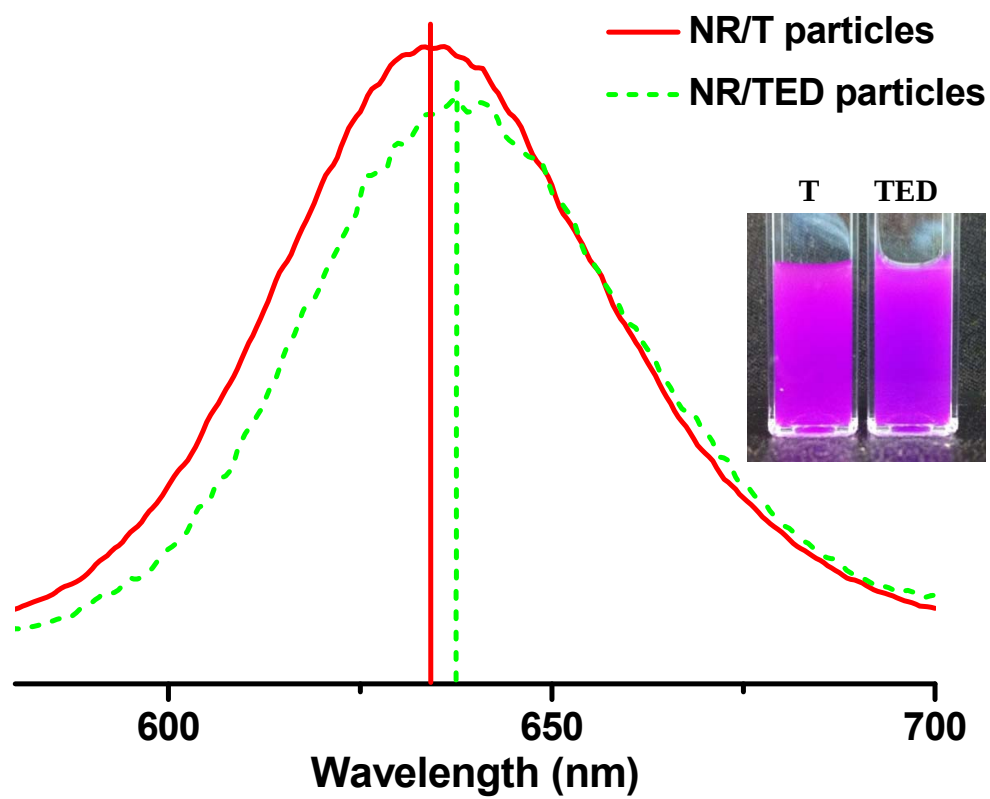


Figure 35 Fluorescence emission intensity of Nile red encapsulated inside T and TED gel particles. The photomicrographs show the appearance of the Nile red loaded gel particle suspensions.

concentration was observed. And the ζ potential increased as a function of reaction time. Using *in vitro* murine macrophage culture, we showed that for short-term treatment, these cationic hydrogel particles were cytocompatible up to different concentration levels, an effect closely related to the positive surface charge on the particles. It was found that particles with a lower positive charge were more cytocompatible than those with a higher positive charge. Furthermore, the capability of drug loading and releasing as a therapeutic carrier was investigated using a hydrophobic dye as the model molecule. Collectively, this novel family of epoxide-polyetheramine based, multi-responsive cationic hydrogel particles offers new opportunities for the delivery of therapeutics such as hydrophobic drugs and genes.

Chapter 3 Synthesis and Characterization of pH and Temperature

Responsive Carboxylated Poly(*N*-vinyl-2-caprolactam)

An article presenting part of the results in this chapter has been published on Journal of Polymer Science Part A: Polymer Chemistry [Tang S., Cao Y., Goddard S. C., He W. Synthesis of 3-(*tert*-Butoxycarbonylmethyl)-*N*-vinyl-2-caprolactam and Homologous Copolymerization Toward Biocompatible Carboxylated Poly(*N*-vinyl-2-caprolactam) Responsive to pH and Temperature. Journal of Polymer Science Part A: Polymer Chemistry 2014; 52: 112-120]. The first author (Shuangcheng Tang) performed the experiments in this study. The corresponding author (Dr. Wei He) is advisor of Shuangcheng Tang and financially supported this work.

3.1 Introduction

Biosensors are very important for gathering *in-situ* information of a patient such as pH and glucose concentrations of blood plasma for better planning therapeutic treatment. A main problem for the long-term functioning of biosensors is the foreign body response including protein adhesion on tip surface in the very beginning and subsequent inflammation in surrounding tissues. To maintain the function of biosensor, it is critical to protect the tip surface from the adsorption of proteins.

Traditionally, the coatings to protect biosensors are realized via grafting a highly-hydrophilic layer composed of PEO structure to reduce the adhesion of proteins [33]. However, the simply hydrophilic protecting layer is far from the demands of the biosensing system. The implantation would cause the variance of targeting parameters,

malfunction of tissue, and death of local cells. Therefore to reduce these effects, control release of therapeutics from the surface layer of the sensor tip was necessary.

The surface layer could be built as multilayer via layer-by-layer self-assembly construction due to the advantages including simplicity and low cost, independence of size and shape of substrate, and the applicability for various polymers [96]. The multilayer is commonly obtained via alternative deposition of two types of polymers using the hydrogen bonding or electrostatic interaction between the neighboring layers. Then the drug molecules can be loaded to the multilayer via covalent bonding or physical interaction. In response to the physiological stimuli the multilayer would swell or desorb to trigger the drug release.

For a higher efficacy of long-term treatment, the desirable release of drug is dependent on the severity of symptom such as the inflammation. The inflammation results in the change of temperature and pH in the local environment which are proper stimuli for the responsive materials to realize control release.

A growing attention has been directed toward *N*-vinyl-2-caprolactam (VCL) (structure shown in Figure 36a) for the preparation of temperature responsive polymers, that is, polymers exhibiting a lower critical solution temperature (LCST) [97], as an alternative, PVCL is more biocompatible than PNIPAM as its hydrolysis will not generate cytotoxic small amide molecules [98]. The LCST of homopolymers of VCL (PVCL) is in the vicinity of physiological temperature, a characteristic favoring biomedical applications [99]. As aforementioned, it is of great significance to introduce pH sensitivity to the thermos-responsive PVCL for a precise control of drug release. To incorporate pH-responsiveness, VCL has been copolymerized with monomers such as

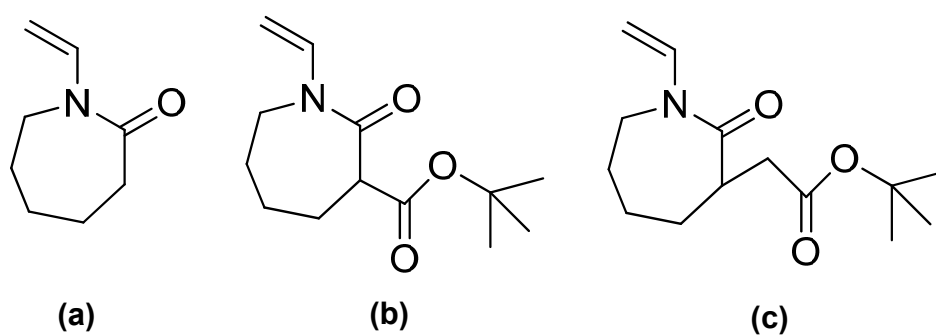


Figure 36 The molecular structure of (a) *N*-vinyl-2-caprolactam (VCL), (b) 3-(*tert* butoxycarbonyl)-*N*-vinyl-2-caprolactam (TBVCL), and (c) 3-(*tert* butoxycarbonylmethyl)-*N*-vinyl-2-caprolactam (TBMVCL).

acrylic acid [100] and acrylamidoglycolic acid [101]. In contrast to such a strategy of adding new heterogeneous monomer units, copolymerization of thermoresponsive monomer with its homologous derivatives offers the advantages of better compositional control and maintaining integrity of the PVCL backbone [102, 103].

Recently, a VCL copolymer was synthesized based on its derivative, 3-(*tert*-butoxycarbonyl)-*N*-vinyl-2-caprolactam (TBVCL), which imparts pH sensitivity to the temperature responsiveness by introducing a protected carboxylic acid substitution group at the 3-position of the lactam ring (Figure 36b) [34]. After the copolymerization of VCL and TBVCL, the carboxylic acid group was exposed via deprotection of the *tert*-butoxycarbonyl (*t*-BOC) group. It was found that the deprotected copolymer was capable of responding to the physiologically relevant pHs ranging from 5.0 to 7.4, with the corresponding LCSTs being 37.5 °C to 44 °C for the 9 mol% carboxyl substituted copolymer, and 40 °C to 64 °C for the 14 mol% substitution. Despite the transitions being pH sensitive, the LCSTs of these copolymers were either at or above physiological temperature at pH 5.0 and 6.0. In order to be useful for drug delivery applications, for example, tissue acidity triggering [12, 104-107], it is desirable to have polymers exhibiting LCST behavior at temperatures below 37 °C when the pH is slightly below 7.4. Since the LCST of a temperature responsive polymer is known to be closely related to its hydrophilicity/hydrophobicity balance, tipping the balance toward more hydrophobic should, in theory, decrease the LCST [108, 109]. Such an approach was exemplified in the work by Yin *et al.*, where with a comparable molar percentage of carboxyl groups, the LCST of the copolymer of NIPAM and methacrylic acid was 36.7 °C at pH 5.0,

whereas the LCST of the copolymer of NIPAM and propylacrylic acid was 23.0 °C at the same pH [11].

Guided by the same principle, here we report the design and synthesis of a more hydrophobic carboxyl substituted VCL derivative, 3-(*tert*-butoxycarbonylmethyl)-*N*-vinyl-2-caprolactam (TBMVCL) (Figure 36c). The major difference between this new monomer TBMVCL and previously reported TBVCL is the hydrophobicity of the substitution group, where in TBMVCL an aliphatic methylene spacing group (-CH₂-) was introduced between the caprolactam ring and the carboxyl group. The monomer was prepared and characterized, and copolymerized with VCL to obtain a series of copolymers with different compositions. Both the protected and deprotected copolymers exhibited LCST behaviors, and the temperature response was also pH dependent for the deprotected copolymers. Furthermore, the chemical nature of the substitution group did influence the LCST. Under a similar degree of carboxylation, the TBMVCL-derived copolymer showed lower LCSTs than the TBVCL-derived copolymer at pH ≤ 5.0, while the opposite trend was observed at pH > 5.0.

Finally, biocompatibility of the carboxylmethyl functionalized PVCL copolymer was demonstrated using NIH/3T3 fibroblast cells. The potential application of the TBMVCL-derived copolymer for surface modification was investigated via layer-by-layer self-assembly with a cationic electrolyte.

3.2 Experimental Section

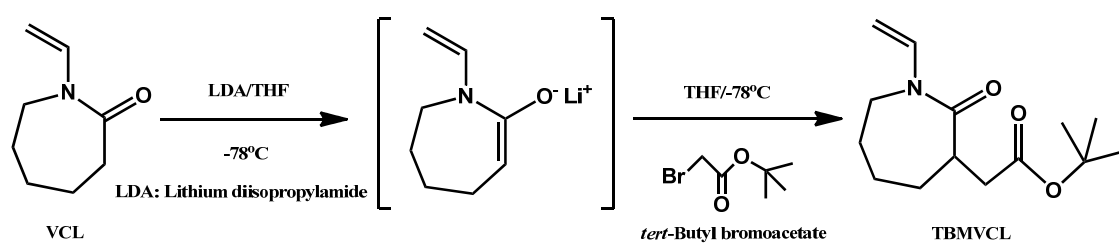
3.2.1 Materials

Azobisisobutyronitrile (AIBN), diisopropylamine (DIPA), *N*-vinyl-2-caprolactam (VCL) were purchased from Sigma-Aldrich. Tetrahydrofuran (THF), *n*-butyllithium,

trifluoroacetic acid (TFA), and *tert*-butyl bromoacetate (TBBA) were obtained from Acros. Deuterated chloroform (CDCl₃) was purchased from Cambridge Isotope Laboratories, Inc. Hexanes, diethyl ether and dichloromethane (DCM) were obtained from Fisher Scientific. Polyethylenimine (Mw 20,000 g/mol) (PEI), polystyrene sulfonic acid (18 wt% in water) (PSS), and polyacrylic acid (PAA) were purchased from Sigma-Aldrich. AIBN was purified via recrystallization in methanol.

3.2.2 Characterization

Mass spectrometry (MS) was performed on an AccuTOF JMS-T100LC MS spectrometer from JEOL, Inc, with the ion source of Direct Analysis in Real Time from ION SENSE, Inc. A Varian Mercury 300 NMR spectrometer (300 MHz) was used to obtain ¹H-NMR and ¹³C-NMR spectra. Fourier transform infrared spectroscopy (FTIR) was performed using sodium chloride plate on a Bio-RAD FTS6000 FTIR spectrometer in a nitrogen atmosphere. The molecular weights of polymers were characterized by gel permeation chromatography (GPC) at room temperature on PL-GPC 50 Plus (Polymer Laboratories, Inc.). *N, N*-dimethylformide was used as the eluting agent and standard monodisperse polystyrenes were used for calibration. Thermogravimetric analysis (TGA) was carried out in a nitrogen atmosphere at a heating rate of 20 °C min⁻¹ on Q-50 (TA Instrument). Potentiometric titration was performed by adding 1 M NaOH solution to a 1 mg mL⁻¹ polymer solution. The pK_a of the polymer was estimated from the corresponding titration curve. The degree of ionization was then calculated as $\alpha = [1/(1 + 10^{(pK_a - pH)})]$.



Scheme 4 Synthesis of monomer 3-(*tert*-butoxycarbonylmethyl)-*N*-vinyl-2-caprolactam (TBMVCL).

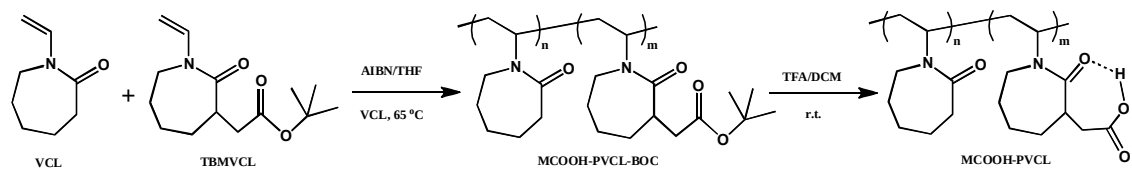
3.2.3 Synthesis of monomer 3-(*tert*-butoxycarbonylmethyl)-N-2-vinyl-caprolactam (TBMVCL)

The preparation approach is presented in Scheme 4. Diisopropylamine (2.02 g, 0.02 mol) was dissolved in 8 mL anhydrous THF. The solution was kept at 0 °C and under nitrogen atmosphere. A 2.5 M solution of *n*-butyllithium (8 mL, 0.02 mol) in hexane was added to the solution in a fashion of dropwise with stirring, then the mixture was cooled down to -78 °C in dry ice bath. VCL (2.78 g, 0.02 mol) was dissolved in 8 mL anhydrous THF, and was added dropwise to the mixture. The resulting solution was maintained at -78 °C for 30 min. TBBA (3.9 g, 0.02 mol) dissolved in anhydrous THF was added in a dropwise fashion to the solution at -78 °C. The reaction mixture was stirred for 2 h at -78 °C and then poured into diethyl ether.

The ether layer was rinsed with water. The organic solution was dried and concentrated by rotovap and purified by column chromatography on silica gel with ethyl acetate/hexane (1/5) as eluent. The desired fractions were combined, concentrated by rotovap and dried in a vacuum oven. The obtained monomer product was named 3-(*tert*-butoxycarbonylmethyl)-N-vinyl-2-caprolactam (TBMVCL). TBMVCL was obtained as a bright yellow viscous liquid with a yield of 70% (MS m/z 254.2 [M+H⁺]).

3.2.4 Synthesis of carboxylated VCL copolymer (MCOOH-PVCL)

In a typical reaction, the copolymerization was followed the synthesis route in Scheme 5. TBMVCL (160 mg, 0.63 mmol), VCL (3 g, 21 mmol), and AIBN (49.2 mg, 0.3 mmol) were dissolved in 10 mL anhydrous THF and transferred into a 35 mL reaction tube. Three freeze-thaw cycles were performed to remove oxygen. Polymerization



Scheme 5 Synthesis of copolymer MCOOH-PVCL-BOCs and deprotected MCOOH-PVCLs.

reaction was carried out at 65 °C for 24 h. The polymer was then precipitated in hexane and dissolved in THF twice for purification. The obtained polymer (MCOOH-PVCL-BOC) was dried by rotovap and then under vacuum at 50 °C. The yields were about 70%. The *tert*-butyl groups were removed via hydrolysis by TFA to recover carboxyl groups. Briefly, MCOOH-PVCL-BOC was dissolved in 10 mL DCM and mixed with excess TFA (20 times in molar ratio to the *tert*-butyl groups). After 3 h stirring at room temperature, the polymer (MCOOH-PVCL) was recovered by precipitation and concentrated by rotovap. The solid product was dried under vacuum at 50 °C.

3.2.5 Cloud point measurement

A 2.5 mg mL⁻¹ polymer aqueous solution was prepared for the measurement. The solution pH was adjusted by 1 M NaOH or 1 M HCl solutions. A UV-vis spectrophotometer (Biomate 5 from Thermospectronic) was used to measure the transmittance of the aqueous polymer solution with a temperature control set. The temperature at 50% light transmittance of solution was defined as the cloud point. A heating/cooling rate of 0.2 °C min⁻¹ was used.

3.2.6 In vitro cytotoxicity study

The cytotoxicity of the MCOOH-PVCL copolymer was tested *in vitro* with NIH/3T3 fibroblast cell line. Cell culture was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Fetal Clone III, Fisher Scientific), 1% L-glutamine and 1% penicillin/streptomycin. Cells were grown at 37 °C, 5% CO₂, and 95% relative humidity. Before cytotoxicity testing, cells were harvested using trypsin-ethylenediamine tetra-acetic acid (EDTA) phosphate buffered saline solution (0.25% trypsin – 0.038% EDTA) and diluted to a density of 1x10⁵ cells mL⁻¹.

The cell suspension was added into 96-well plates at 100 μL well⁻¹, and the cells allowed attaching overnight. Following this incubation period, culture medium was removed and replaced with fresh medium containing MCOOH-PVCL 9 at various concentrations between 0.02 and 2.5 mg mL⁻¹. Following overnight incubation, cellular cytotoxicity was evaluated with a WST-1 assay (Roche, Cell Proliferation Assay) according to the manufacturer's instruction. Negative (without MCOOH-PVCL 9) and blank (without cells) controls were also included. Statistical analysis was conducted using Student's *t*-test.

3.2.7 Layer-by-layer self-assembly of MCOOH-PVCL 19

For the multilayer construction via layer-by-layer self-assembly, the electrostatic interaction was employed. The copolymer MCOOH-PVCL 19 with carboxylic acid groups is deprotonated and negatively charged at pH 7.4, as well as the PSS. On the contrary, the PEI is protonated and positively charged at pH 7.4. The MCOOH-PVCL 19, PEI, and PSS were dissolved in 0.1 M phosphate buffered saline (PBS) to prepare solutions of 1.0 mg/mL at pH 7.4 respectively. A quartz crystal microbalance setup (QCM) with dissipation detector, QCM-D (Q-Sense E4 System, Biolin Scientific, Sweden) with gold-coated silica quartz crystal sensors was applied to investigate the construction and responsive behavior of multilayers composed of oppositely charged PEI/MCOOH-PVCL 19. To ensure the appropriate adsorption of PEI and the efficient deposition of MCOOH-PVCL 19, two bilayers of PEI/PSS was built before constructing bilayers of MCOOH-PVCL 19/PEI. The multilayers were initially fabricated by the sequential and alternative flowing the PEI and PSS solutions through the QCM chamber at 0.15 mL/min. After the chamber being filled with the target solution, the flow was

stopped for 10 min allowing the deposition. Between the PEI and PSS solutions, PBS solution was flowed through for 10 min to rinse the chamber. Two bilayers of PEI/PSS were built and capped with an additional layer of PEI for subsequent construction of 5 bilayers of MCOOH-PVCL 19/PEI. The total thickness of the two PEI/PSS and one PEI layers was 5 nm. The gold-coated silica sensors with a fundamental resonance frequency of 4.95 MHz were cleaned using piranha solution. The shifts in the resonance frequency (Δf_n) of the sensors were obtained at multiple harmonics (overtone number $n=1, 3, 5, 7, 9, 11$, and 13) as well as the energy dissipation factor (ΔD_n) via energy decayed after the driving power was switched off [110].

The Δm (adsorbed mass) was obtained via the Sauerbrey equation [111]

$$\Delta m = \frac{1}{n} \frac{t_q \rho_q}{f_0} \Delta f$$

where t_q is the thickness of quartz ($\sim 330 \mu\text{m}$), ρ_q is the density of quartz (2.684 g/cm^3), f_0 is the fundamental resonance frequency (4.95 MHz). The thickness was estimated using adsorbed mass.

The dissipation was calculated via the following equation [110]:

$$\Delta D = \frac{E_{Dissipated}}{2\pi E_{Stored}}$$

where the $E_{Dissipated}$ is the decayed energy per oscillation and the E_{Stored} is the energy stored per oscillation.

The thermal responsiveness of the multilayers was investigated via adjusting the temperature of the chamber and subsequent incubating 30 min without flowing for equilibrium. The room temperature (22°C) and physiological temperature (37°C) were

studied at the pH 2, pH 5, and pH 7.4. For determination of pH sensitivity, the PBS solutions at different pH were being flowed through the chamber for 30 min followed by 10 min incubating. The responses to acidic stimuli were focused while the pH was varied as 1, 2, 3, 4, 5, 6, and 7.4.

3.3 Results and Discussions

3.3.1 Monomer synthesis and characterization

The new VCL monomer derivative, TBMVCL, was designed to differ from the previously reported TBVCL monomer in the extent of proximity of the protected carboxyl group to the lactam ring. In TBVCL, the group was directly linked to the ring, whereas in TBMVCL, the group was indirectly linked via an aliphatic methylene spacing unit. Synthesis of the TBMVCL monomer followed the general substitution mechanism in functionalizing *N*-vinyl lactams [34, 112-116]. As shown in Scheme 4, VCL was first activated to an enolate intermediate in the presence of a base lithium diisopropylamide (LDA). The VCL carbanion obtained was then reacted with *tert*-butyl bromoacetate to form TBMVCL in a good yield (~70%) via nucleophilic substitution at the α -carbon to the lactam carbonyl group.

The new monomer was thoroughly characterized by NMR and FTIR. Specifically, the chemical structure of TBMVCL was confirmed by the ^1H -NMR spectrum (Figure 37a). The peaks at chemical shift 7.3 ppm and 4.4 ppm can be assigned to the methine and methylene protons of the vinyl group, respectively. Successful incorporation of the *tert*-butoxy carbonylmethyl group was supported by the appearance of two doublets of doublets peaks at 2.19-2.30 ppm and 2.77-2.88 ppm, which corresponded to the methylene protons, along with a sharp singlet at 1.38 ppm originating from the *tert*-butyl

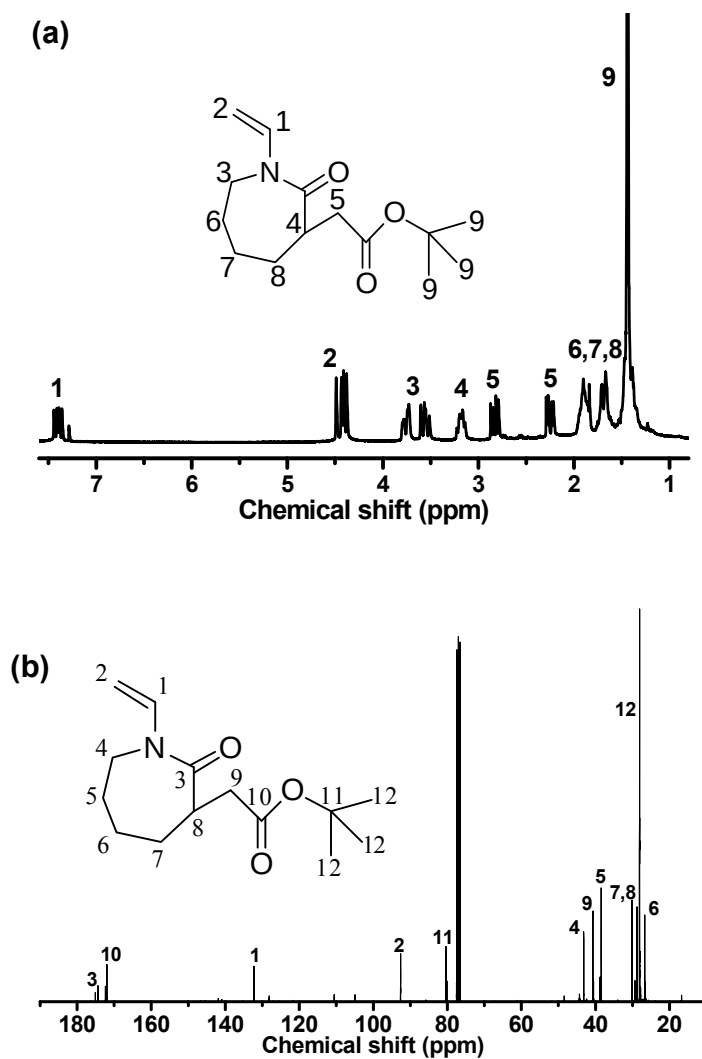


Figure 37 ^1H -NMR spectrum (a) and ^{13}C NMR spectrum (b) of monomer TBMVCL in CDCl_3

protons. ^{13}C -NMR spectrum (Figure 37b) revealed 12 different peaks corresponding to 12 different carbons in the monomer structure. The ester carbonyl carbon from the substitution group was clearly observed at 172 ppm. In the FTIR spectrum (Figure 38), the strong absorption at 1735 cm^{-1} is due to the C=O stretching vibration of the ester group. The absorption at 1660 cm^{-1} is caused by the C=O stretching vibration of the lactam amide group, while the peak at 1650 cm^{-1} arises from the C=C stretching vibration of the vinyl group. Collectively, these results confirmed the success of synthesis of the new VCL monomer derivative.

3.3.2 Copolymer MCOOH-PVCL-BOCs synthesis and characterization

As pointed out earlier, the motivation of synthesizing a new TBMVCL monomer was to study the side group effect on the thermos-responsiveness of VCL-based polymers. Therefore, a series of copolymers were prepared by polymerizing TBMVCL with VCL via conventional AIBN initiated free radical solution polymerization (Scheme 5) by varying the molar feeding percentages of TBMVCL from 3% to 17%.

The molecular weights of the obtained copolymers were characterized by GPC. As summarized in Table 2, the number average molecular weights of these MCOOH-PVCL-BOC copolymers range from 3,800 g/mol to 4,800 g/mol, and the molecular weight distribution is broad, a characteristic typical of AIBN free radical solution polymerization. It is important that these copolymers have comparable molecular weights, as studies have shown LCSTs of VCL polymers being dependent on molecular weights [115].

The chemical compositions of the copolymers were determined by TGA measurements. As shown in Figure 39, at temperatures higher than $210\text{ }^{\circ}\text{C}$, the *t*-BOC

Table 2 Molecular weight and compositional data of copolymers

Polymer	TBMVCL amount [mol%]		$\bar{M}_n$ [g mol ⁻¹]	$\bar{M}_w / \bar{M}_n$
	In feed	In polymer ^a		
MCOOH-PVCL-BOC 2	3	2.2	4,800	2.8
MCOOH-PVCL-BOC 9	10	8.8	3,800	1.7
MCOOH-PVCL-BOC 19	17	18.6	4,700	1.7

^aEstimated from TGA

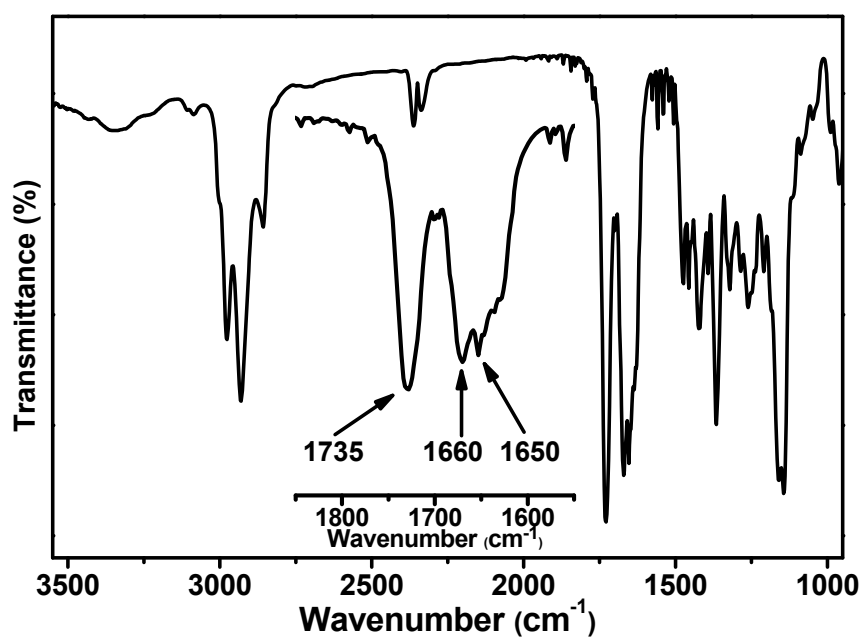


Figure 38 FTIR spectrum of monomer TBMVCL.

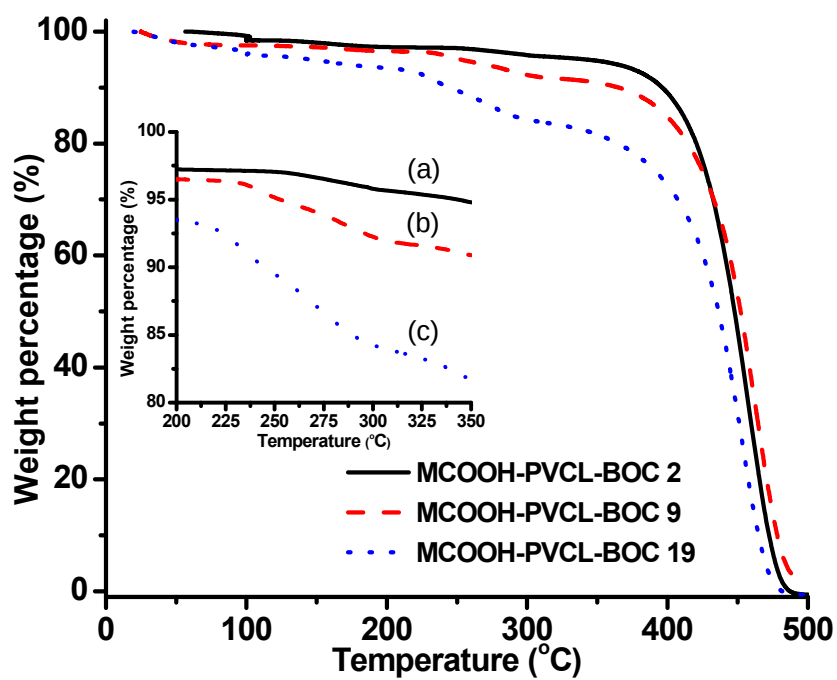


Figure 39 TGA curves of (a) MCOOH-PVCL-BOC 2, (b) MCOOH-PVCL-BOC 9, and (c) MCOOH-PVCL-BOC 19.

groups undergo spontaneous thermolysis into gas phase of carbon dioxide (CO₂) and isobutylene (C₄H₈) [117]. The corresponding weight loss can then be used to calculate the molar content of TBMVCL in the copolymers, which was found to be 2.2%, 8.8%, and 18.6% respectively for the three copolymers. The actual compositions of the copolymers being close to the feed ratio suggest that the reactivity of monomer TBMVCL is similar to that of VCL. In contrast, our previous study showed the reactivity of TBVCL being higher than VCL [34]. Such a difference can be attributed to the newly added methylene spacing group in TBMVCL, which reduce the polarity factor of the monomer. Comparing with the *tert*-butoxycarbonyl group, the *tert*-butoxycarbonylmethyl substitution group reduced the electronegativity of the α -carbon of the lactam ring, and resulted in a less reactive monomer TBMVCL [118-120].

The consistency between the final composition and the feeding ratio could be explained using the reactivity ratio of each monomer as well. To predict reactivity ratio, Alfrey and Price developed the Q-e scheme for free radical copolymerization [118]. The Q represents the resonance effect while the e represents the polar property of the monomer. The reactivity ratio of VCL (r_1) can be obtained using the equation below:

$$r_1 = \left(\frac{Q_1}{Q_2} \right) e^{-e_1(e_1 - e_2)}$$

The reactivity ratio of TBMVCL (r_2) can be obtained using the equation below:

$$r_2 = \left(\frac{Q_2}{Q_1} \right) e^{-e_2(e_2 - e_1)}$$

Here the Q and e of VCL are 0.14 and -1.18, respectively, as reported in the

literature (<http://www.plastics-material.com/q-e>). The *tert*-butoxycarbonylmethyl substitution group in TBMVCL enhanced electron-donating effect of the lactam ring in comparison with the VCL. So the Q value of TBMVCL is lower than that of VCL as well as the e value. It was observed in the calculation that the r_1 and r_2 are very close while the r_2 is slightly lower. The result suggested that based on the structure, the reactivity of TBMVCL is slightly lower than that of VCL.

The polymer structure and functional groups were examined by ^1H NMR and FTIR. Figure 40 showed the ^1H -NMR spectra of the MCOOH-PVCL-BOC copolymers with different compositions. Firstly, the absence of vinylic signals suggested that the monomers were successfully polymerized. Secondly, the signal of *tert*-butyl protons at 1.38 ppm indicated successful incorporation of TBMVCL in the copolymer. The intensity of this peak was found to increase (Figure 40a-c) as the molar feeding percentage of TBMVCL increased. As a representative example, the FTIR spectrum of the copolymer MCOOH-PVCL-BOC 19 was shown in Figure 41a. The strong peak at 1641 cm^{-1} belonged to the characteristic C=O stretching vibration absorption associated with the amide group from the caprolactam moiety. The peak at 1726 cm^{-1} originated from ester C=O stretching vibration absorption, a further proof of the presence of TBMVCL units in the copolymer.

In order to enable the study of pH-dependent thermal behaviour, the MCOOH-PVCL-BOC copolymers were treated with TFA to hydrolyze the *t*-BOC groups into carboxylic acid groups. The structures of the hydrolyzed products MCOOH-PVCLs were characterized by ^1H -NMR and FTIR. As shown in Figure 42, the signal of *tert*-butyl protons at 1.38 ppm originally observed in the protected copolymer (Figure 40) has

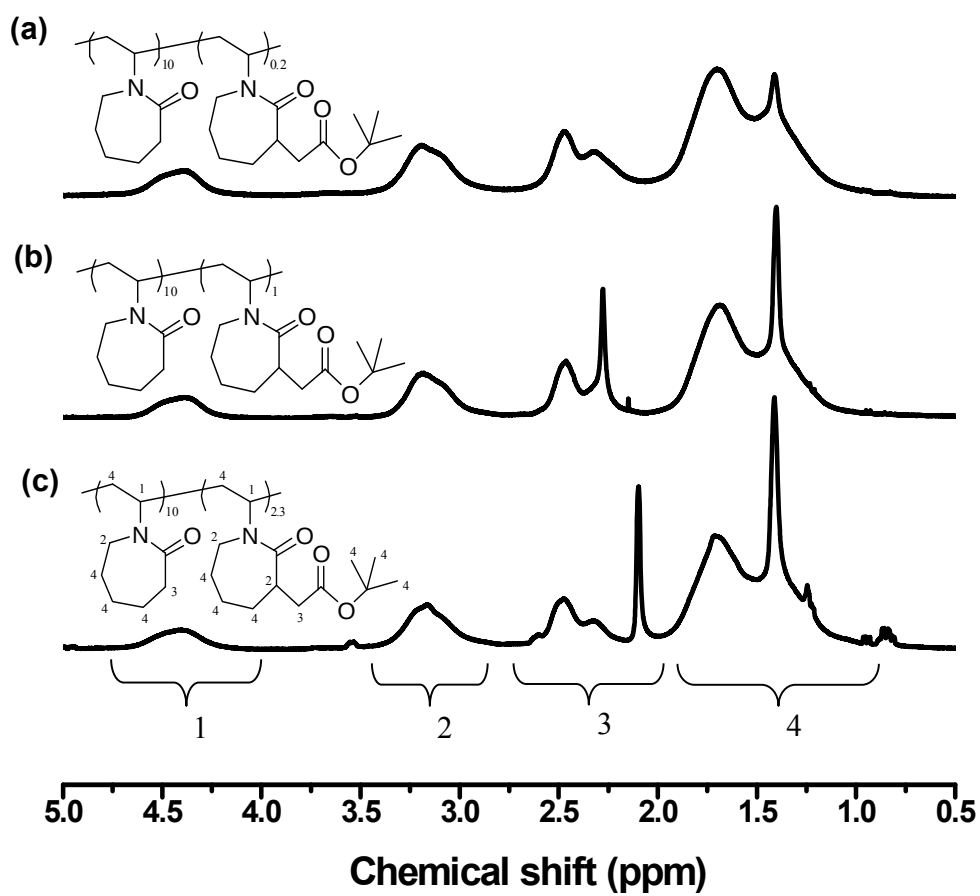


Figure 40 ^1H -NMR spectra of protected copolymer (a) MCOOH-PVCL-BOC 2, (b) MCOOH-PVCL-BOC 9, and (c) MCOOH-PVCL-BOC 19 in CDCl_3 .

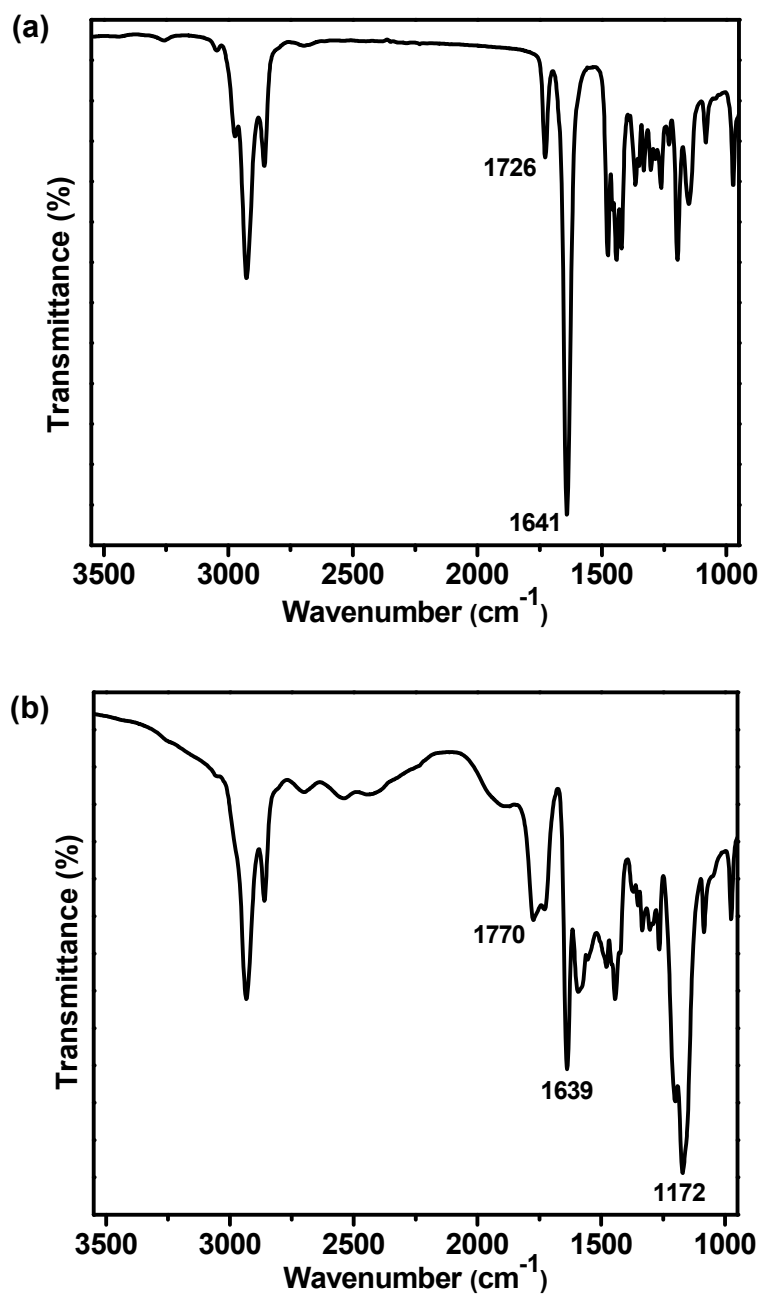


Figure 41 FTIR spectra of (a) protected copolymer MCOOH-PVCL-BOC 19 and (b) deprotected copolymer MCOOH-PVCL 19.

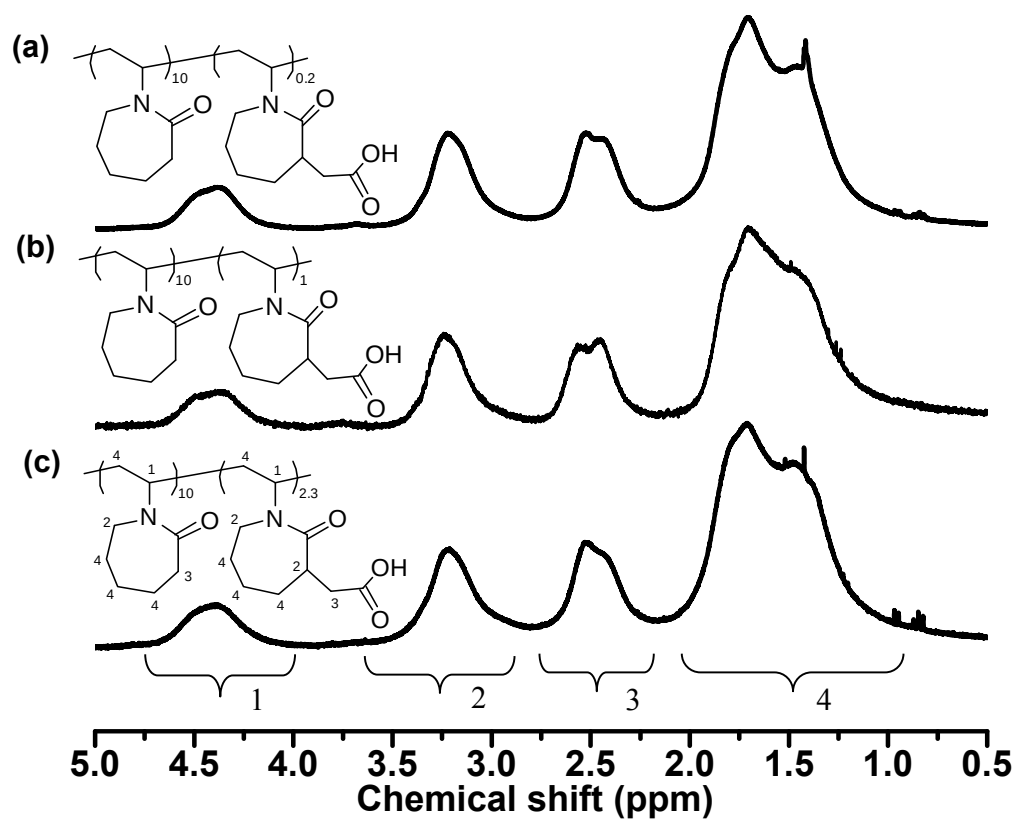


Figure 42 ^1H -NMR spectra of deprotected copolymer (a) MCOOH-PVCL 2, (b) MCOOH-PVCL 9, and (c) MCOOH-PVCL 19 in CDCl_3 .

disappeared. The success of deprotection was also observed in the FTIR spectrum (Figure 41b) where the broad absorbance between 2500 and 3500 cm^{-1} (-OH stretching) demonstrated the existence of -COOH groups in the copolymer.

Potentiometric titration was performed on the MCOOH-PVCL copolymers for dissociation behaviour study and polymer acidity characterization. Due to the high content of -COOH groups, the MCOOH-PVCL 19 was only partially soluble at room temperature.

Therefore, we focused the titration study on copolymers MCOOH-PVCL 2 and MCOOH-PVCL 9. The overall shape of the titration curve (Figure 43) bears a close resemblance to that of a weak acid [121-123]. As the degree of ionization increases, the solution pH increases steadily. The pK_a values of MCOOH-PVCL 2 and MCOOH-PVCL 9 were determined to be 3.2 and 3.4, respectively. The higher pK_a observed for the copolymer with a higher molar content of carboxyl groups can be explained by the “polyelectrolyte effect”, a phenomenon also noted in aqueous solutions of other types of carboxylic acid containing polymers, such as poly(*N*-vinyl-2-pyrrolidone-*co*-acrylic acid) [124] and poly(*N*-vinylacetamide-*co*-acrylic acid) [125].

3.3.3 LCST behaviours of MCOOH-PVCL-BOCs and MCOOH-PVCLs

We studied the thermoresponsive behaviors of the copolymers both before and after deprotection using a common turbidimetric approach. For the protected copolymers, despite bearing the bulky and hydrophobic *tert*-butyl group, within the compositions studied the copolymers were soluble in water to various extents, allowing us to carry out cloud point measurements. As shown in Figure 44a, all three copolymers were thermal responsive and the solution phase transition profiles were dependent on the copolymer

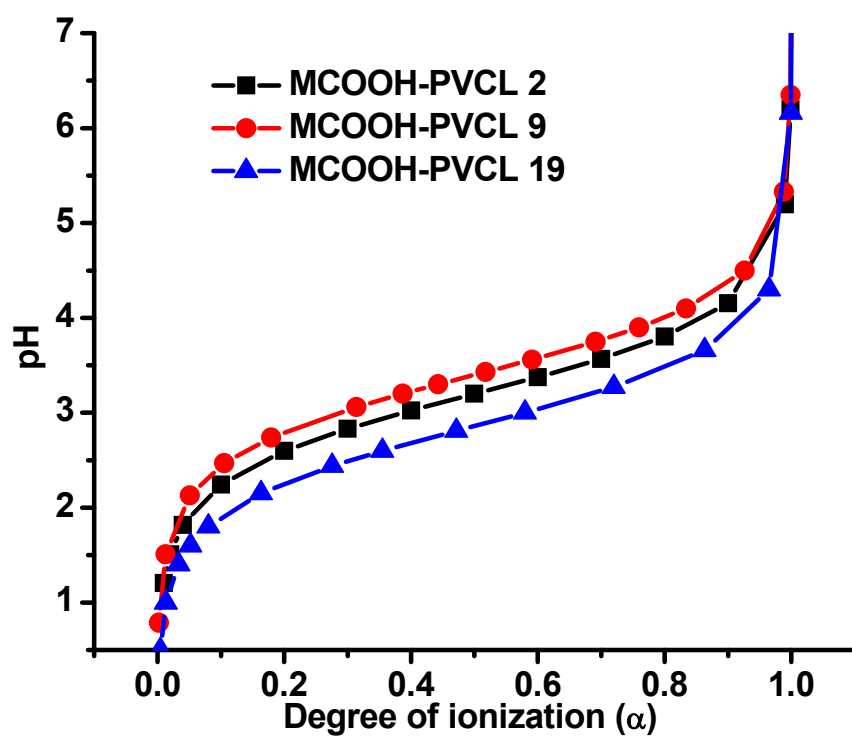


Figure 43 pH versus degree of ionization for MCOOH-PVCL copolymer solutions.

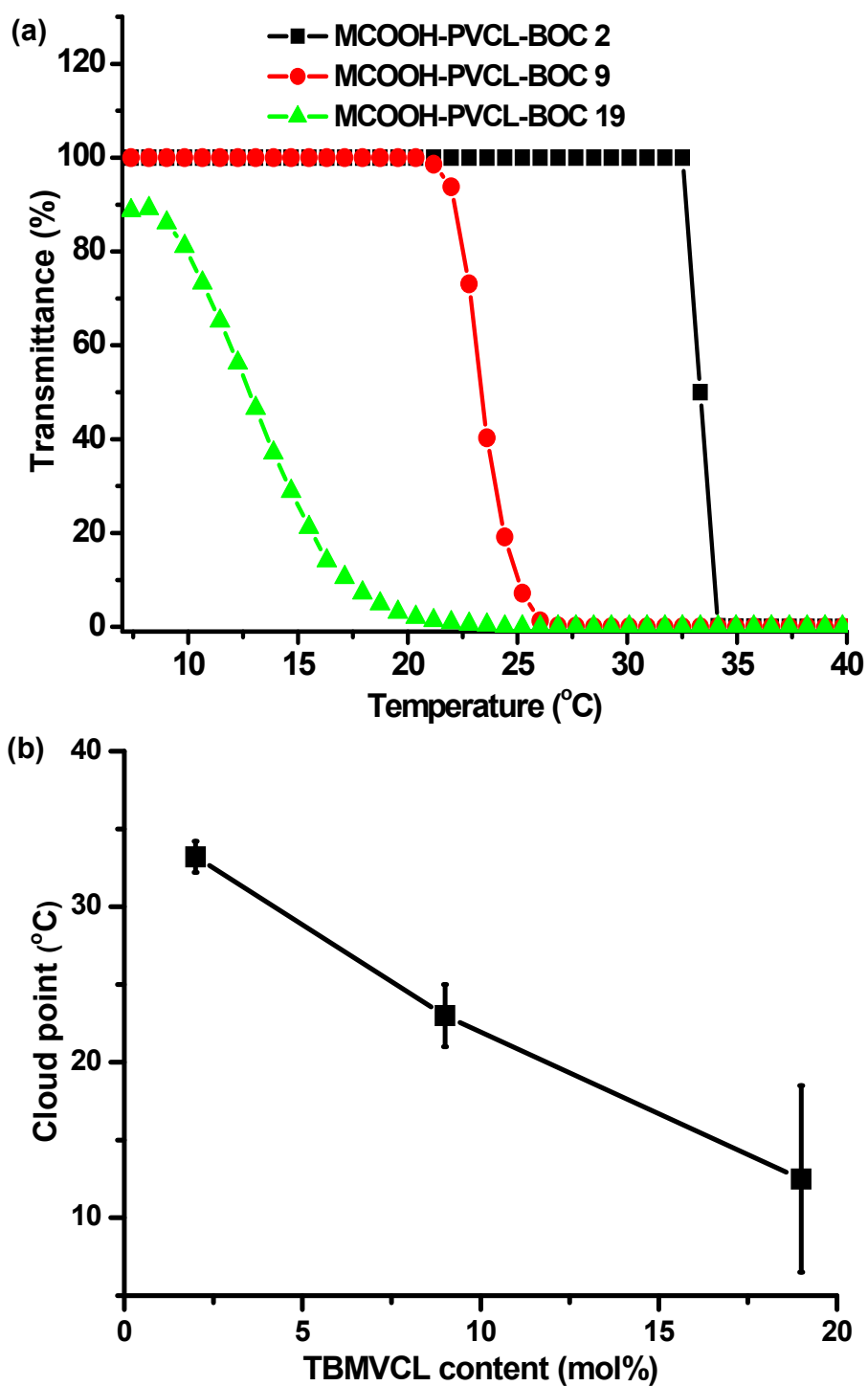


Figure 44 (a) Phase transition profiles of various MCOOH-PVCL-BOC copolymer solutions. (b) The dependence of MCOOH-PVCL-BOC cloud points on copolymer compositions.

molar composition. First of all, as summarized in Figure 44b, the cloud point, defined as the temperature at which the transmittance of a polymer solution reaches 50% of the initial value, decreased from 33 °C to 13 °C when the TBMVCL composition increased from 2.2 mol% to 18.6 mol%. This is due to the fact that TBMVCL is more hydrophobic than VCL. Therefore, a higher TBMVCL content will result in an increase of the overall hydrophobicity of the copolymer, leading to a lower LCST. Such an influence is also reflected by the initial transmittance of MCOOH-PVCL-BOC 19 solution being 90%, indicating an incomplete solvation of this copolymer by water molecules. The other observation is the shape of the phase transition profile (Figure 44a). While the MCOOH-PVCL-BOC 2 and MCOOH-PVCL-BOC 9 exhibited a sharp transition upon heating, the transition shown by MCOOH-PVCL-BOC 19 was more gradual. We attributed the difference in thermal response sensitivity to the bulky hydrophobic *tert*-butyl side group. It is well known that the macroscopic change in the solution turbidity of thermal responsive polymers is a reflection of the polymer chain undergoing a coil-globule transition at the molecular level and subsequent aggregation of the globules as the solution temperature increases [126-130]. The presence of bulky hydrophobic side groups could change the chain association behavior. With 18.6 mol% of TBMVCL units in the copolymer, the *tert*-butyl groups could interact with VCL units, inducing dehydration of these VCL units even at temperatures lower than the phase transition and resulting in hydrophobic associations between VCL and *tert*-butyl groups. This explains the observed gradual increase in solution turbidity for MCOOH-PVCL-BOC 19 as the temperature rises (Figure 44a). Such an impact was negligible when the amount of TBMVCL in the copolymer was lower than 10 mol%. Our results correlated well with the study by

Wintgens and Amiel, where thermoresponsive PNIPAM was hydrophobically modified with bulky adamantyl group. It was observed that sharp transitions occurred in the case of PNIPAMs with low substitution levels (1.4%), in contrast to the broader transitions for PNIPAMs with high substitution levels (4.5%) [131]. Removal of the *tert*-butyl protecting groups via acid hydrolysis exposed the carboxyl groups, and the resulting deprotected copolymers exhibited pH-dependent thermal response. It is well known that the underlying mechanism is closely related to the protonation-deprotonation of the carboxyl groups as pH varies, leading to changes in the overall hydrophobicity/hydrophilicity of the polymers [132]. As shown in Figure 45, the phase transition behaviors of MCOOH-PVCL copolymers were compared at different solution pH. A strong influence of the copolymer composition on the thermoresponsive behavior was observed. Bearing the lowest amount of carboxyl groups, the MCOOH-PVCL 2 exhibited a relative narrow range of cloud points, from 33 °C at pH 2.0 to 44 °C at pH 7.4. A moderate increase in the molar content of carboxyl groups resulted in a drastic change in the range of cloud points. For MCOOH-PVCL 9, its solution cloud point ranges from 27 °C at pH 2.0 to 64 °C at pH 6.0, and the optical transmittance of the pH 7.4 solution at 75 °C is ~70%. For MCOOH-PVCL 19, which contains the highest molar fraction of carboxyl groups, an even broader range of cloud points was observed from pH 2.0 (16 °C) to pH 6.0 (66 °C), and no cloudiness was detected for the pH 7.4 solution within the upper temperature limit of the equipment. The polymer compositional effect on cloud points can be correlated with its impact on polymer hydrophobicity and its affinity factor χ with the water molecules. For example, at pH 2, the carboxyl groups of the copolymers

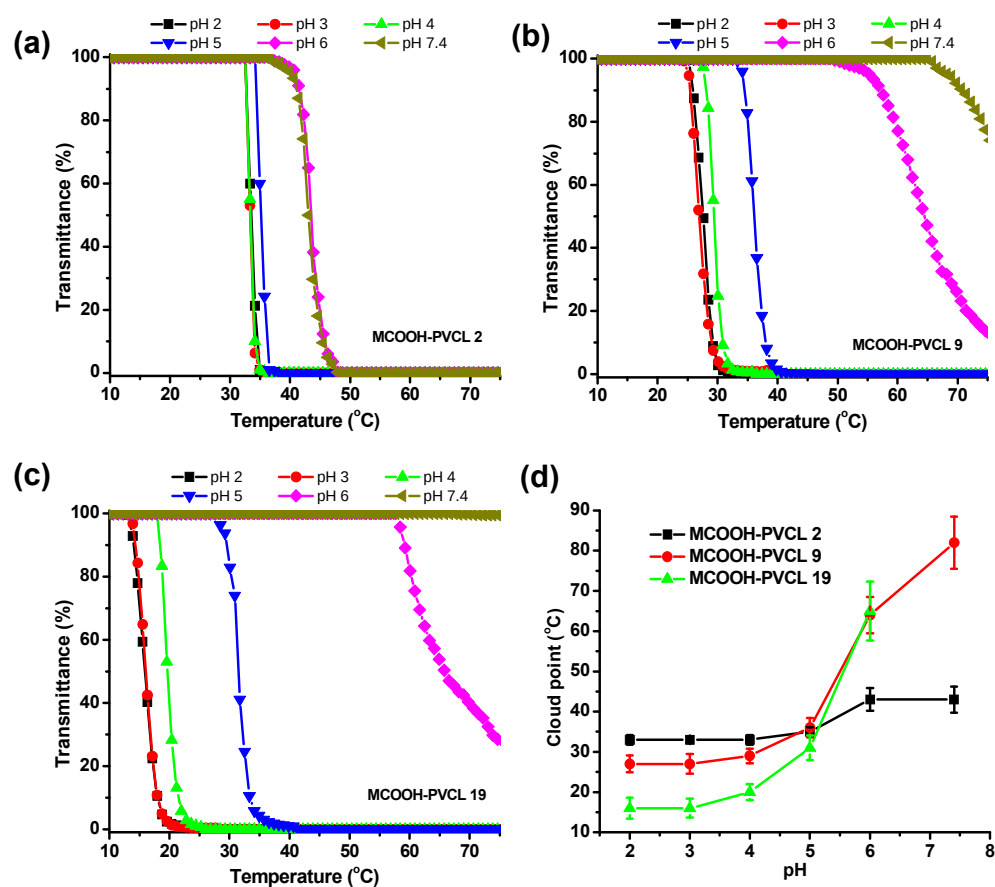


Figure 45 Phase transition profiles of deprotected copolymers (a) MCOOH-PVCL 2, (b) MCOOH-PVCL 9, (c) MCOOH-PVCL 19 at various pH. (d) The cloud points for each copolymer were summarized as a function of pH.

were protonated to the hydrophobic carboxylate form (-COOH) resulting the increase of χ and corresponding decrease of miscibility between the copolymer and solvent. Therefore, MCOOH-PVCL 19 had the lowest cloud point at this pH due to it being the most hydrophobic among the three polymer compositions. In contrast, ionization of the -COOH groups at pH 7.4 led to increase of polymer hydrophilicity and decrease of χ . As a consequence, a higher temperature was required for phase separation.

To examine the influence of ionic strength on the LCST behaviors of the deprotected copolymers, we also measured the cloud points of MCOOH-PVCL 9 dissolved in PBS with a physiologically relevant salt concentration (i.e. 150 mM). It was found that the cloud points of the copolymer in salt solution were lower than those in water, and the effect was more apparent at high pH (Figure 46). Such a suppression effect by salts has been widely studied for both non-ionic [68, 133, 134] and ionic [132] thermoresponsive polymers. It is commonly referred to as a salting out effect, where the salt ions compete for the water molecules available for hydration, leading to phase separation of the polymer occurring at a lower temperature [135].

The influence of polymer solution concentration on the phase transition was also studied using the 8.8 mol% copolymer (MCOOH-PVCL 9) as an example with the solution pH limited to 5.0. The phase transition curves were shown in Figure 47a with polymer concentrations ranging from 0.25 to 7.5 mg mL⁻¹. It is clear that increasing polymer concentration lowers the cloud point by more than 20 °C (Figure 47b) and also sharpens the transition. Such significant concentration dependence can be explained by the role of intermolecular chain interactions in thermally induced phase separation [69]. Increase of polymer concentration will increase the probability of chain-chain

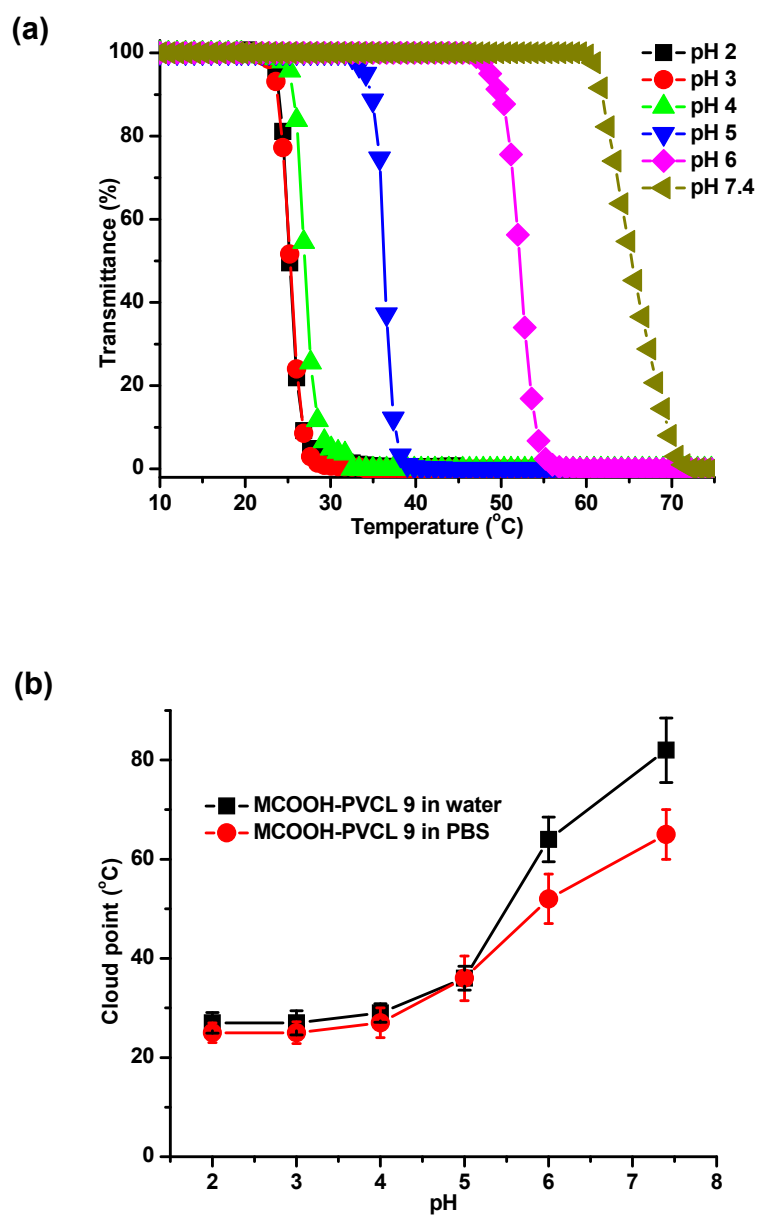


Figure 46 (a) Phase transition profiles of MCOOH-PVCL 9 (2.5 mg/mL in 0.01 M phosphate buffered saline (PBS)) and (b) the cloud points of MCOOH-PVCL 9 in water and in PBS were summarized as a function of pH.

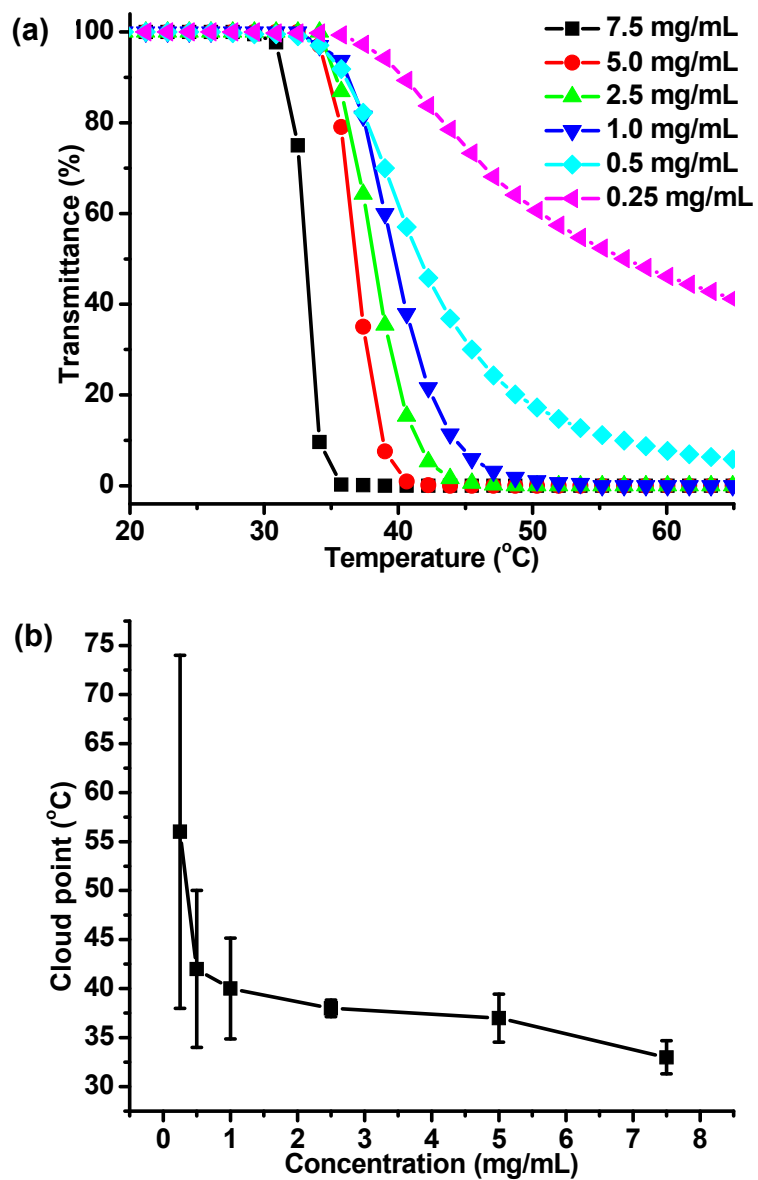


Figure 47 (a) Polymer concentration dependence of phase transition profiles for MCOOH-PVCL 9 solution at pH 5. (b) The effect of polymer concentration on the cloud points of MCOOH-PVCL 9 solution at pH 5.

interactions, allowing phase transition to occur at a lower temperature. The lowest concentration of 0.25 mg mL^{-1} showed a very diffuse and incomplete transition, in which the solution did not turn fully opaque, suggesting inhibited chain collapse and insufficient polymer aggregate formation. Similar concentration effects on the phase transition temperature have also been reported for other thermoresponsive polymers [136-138].

Another aspect of relevance to thermoresponsive polymers is the reversibility of the phase transition. By monitoring the phase transition profiles of MCOOH-PVCL 9 (pH 5.0 solution) during the heating and subsequent cooling cycle, we observed a minor degree of thermal hysteresis (Figure 48). The cloud point shown from the cooling curve was 1.5°C lower than that determined by the heating curve. The occurrence of hysteresis could be accounted for by a couple of factors: (1) formation of interchain hydrogen bonding enabled by the carboxyl groups, which is similar to the reason behind thermal hysteresis for PNIPAM [139, 140]; and (2) water bound to collapsed polymer globules even at temperatures above LCST, a mechanism reported in a recent study on temperature-induced phase separation of PVCL by Spěvák et al. [141].

3.3.4 Comparisons of LCST behaviors of MCOOH-PVCL with COOH-PVCL copolymers

As mentioned earlier, the main motivation of introducing an additional methylene group between the carboxylic acid group and the caprolactam ring is to increase the hydrophobicity of the polymer and reduce the cloud points of the copolymers. To examine this hypothesis, cloud points of MCOOH-PVCL 9 synthesized in the current study and those of COOH-PVCL 9 reported in our previous work were summarized in Figure 49 with regard to various pH. Given the same molar composition ($\sim 9\%$) of

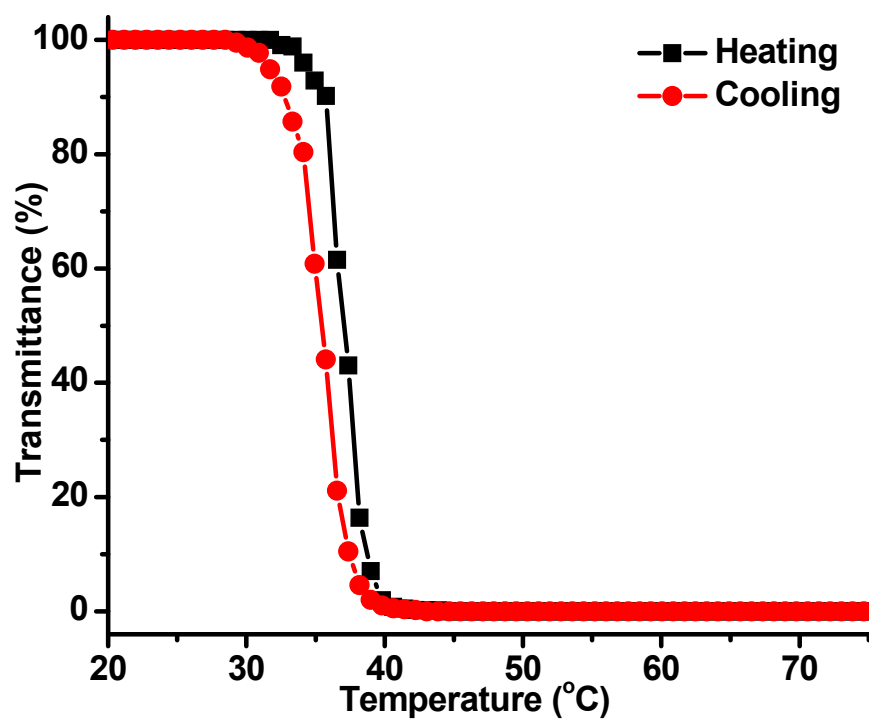


Figure 48 Heating and cooling phase transition profiles of MCOOH-PVCL 9 phosphate buffered saline solution (pH = 5).

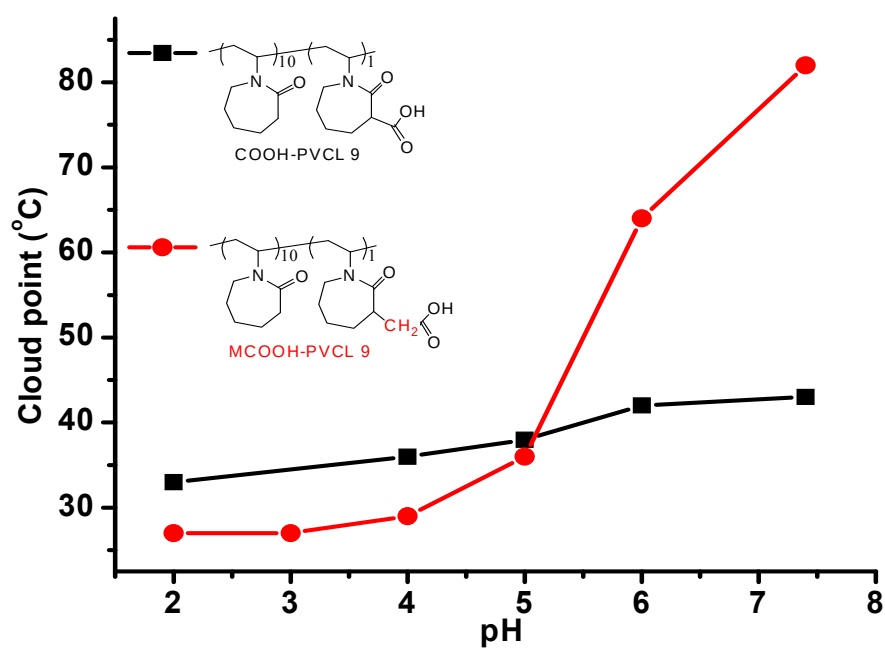


Figure 49 Comparison of cloud points of COOH-PVCL 9 and MCOOH-PVCL 9 at different pH values.

carboxylic acid group in both copolymers, we could focus on the effect of monomer structure on LCST. At $\text{pH} \leq 5.0$, the cloud points of MCOOH-PVCL 9 were lower than those of COOH-PVCL 9. However, above pH 5, the cloud points of MCOOH-PVCL 9 are higher than those of COOH-PVCL 9, which was opposite to our expectation.

Such a paradox behavior may be due to the steric hindrance effect of the additional methylene group. Even though the carboxylmethyl group ($-\text{CH}_2\text{COOH}$) carries weaker acidity than the carboxyl group ($-\text{COOH}$), the methylene spacing unit may add difficulty for hydrophobic chain-chain association under ionizing solution pH. A similar steric effect has been reported on non-ionic thermoresponsive polymers, where poly(*N*-isopropylmethacrylamide) (PNIPMAM) had a higher LCST than PNIPAM, despite the fact that PNIPMAM was more hydrophobic than PNIPAM [142]. Further investigations on the phase separation and hydration of MCOOH-PVCLs may help elucidate the precise effects of addition of hydrophobic spacing groups.

3.3.5 Cytotoxicity of MCOOH-PVCLs

In addition to thermal behaviours, biocompatibility of the new PVCL copolymers was also evaluated. Using a fibroblastic cell line, we examined the cytotoxicity of MCOOH-PVCL 9 with a concentration ranging from 0.02 to 2.5 mg mL^{-1} . The upper limit was selected based on our previous cell study with COOH-PVCL, where a significant reduction in cell viability was observed for cells treated with 5 mg mL^{-1} polymers [34]. The *in vitro* WST-1 assay results (Figure 50) showed that the MCOOH-PVCL 9 copolymer was nontoxic to cells at concentrations below 2.5 mg mL^{-1} . The mild decrease in viability at a polymer concentration of 2.5 mg mL^{-1} can be attributed to slight acidification of the culture medium by the carboxylated polymer.

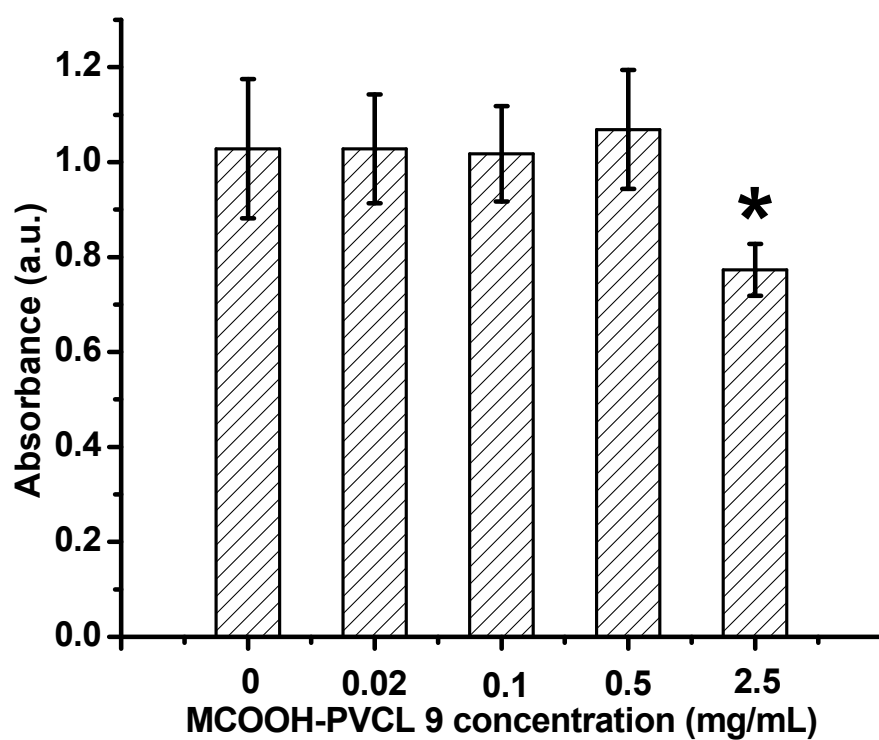


Figure 50 NIH/3T3 fibroblast cells viability after overnight incubation with different concentrations of MCOOH-PVCL 9 solutions. Data shown are average $\pm$ standard deviation (STD) ($n = 3$; * $p < 0.01$).

3.3.6 Layer-by-layer self-assembly of MCOOH-PVCL 19

In order to fabricate responsive surface for delivery of biomedical therapeutics and bio-molecules including drugs, proteins and nucleic acids [143], the multilayers were built using the copolymer MCOOH-PVCL 19. There are mainly two approaches available for multilayers construction, including hydrogen bonding and electrostatic interaction, considering the nature of MCOOH-PVCL 19 with carboxylic acid groups. It is preferred to perform layer-by-layer self-assembly under mild condition (pH 7.4 and room or physiological temperature). At pH 7.4, the copolymer is deprotonated considering that the pK_a of MCOOH-PVCL 19 is around 3. For a negatively charged electrolyte, it is straightforward to build multilayers via electrostatic interaction.

Here in this system, the PEI, with pK_b around 8.8 [144], is used as the positively charged polymer which is protonated at pH 7.4 in PBS (Figure 51a). The successful construction of multilayers was proved by the frequency shift Δf as a function of number of layers as shown in Figure 52a. The Δf of 5 bilayers was -230 Hz which represented increase of 40 nm (Figure 52b). The thickness of all 5 bilayers are quite homogeneous at 8 nm with no obvious trend of exponential growth as a function of number of layers. The polyelectrolytes deposited on the layer of oppositely charged polymer resulting a compensation of charges at the interface [145]. However, over-compensation occurred and benefited the deposition of the next layer [146]. In the linearly growing multilayers, each layer directly contacts and electrostatically interacts only with its neighbouring layers, suggesting an orderly structure of layers [147, 148].

In each bilayer, it is observed that MCOOH-PVCL 19 contributed the majority portion of mass or thickness increase while it seemed the deposited PEI is of very low

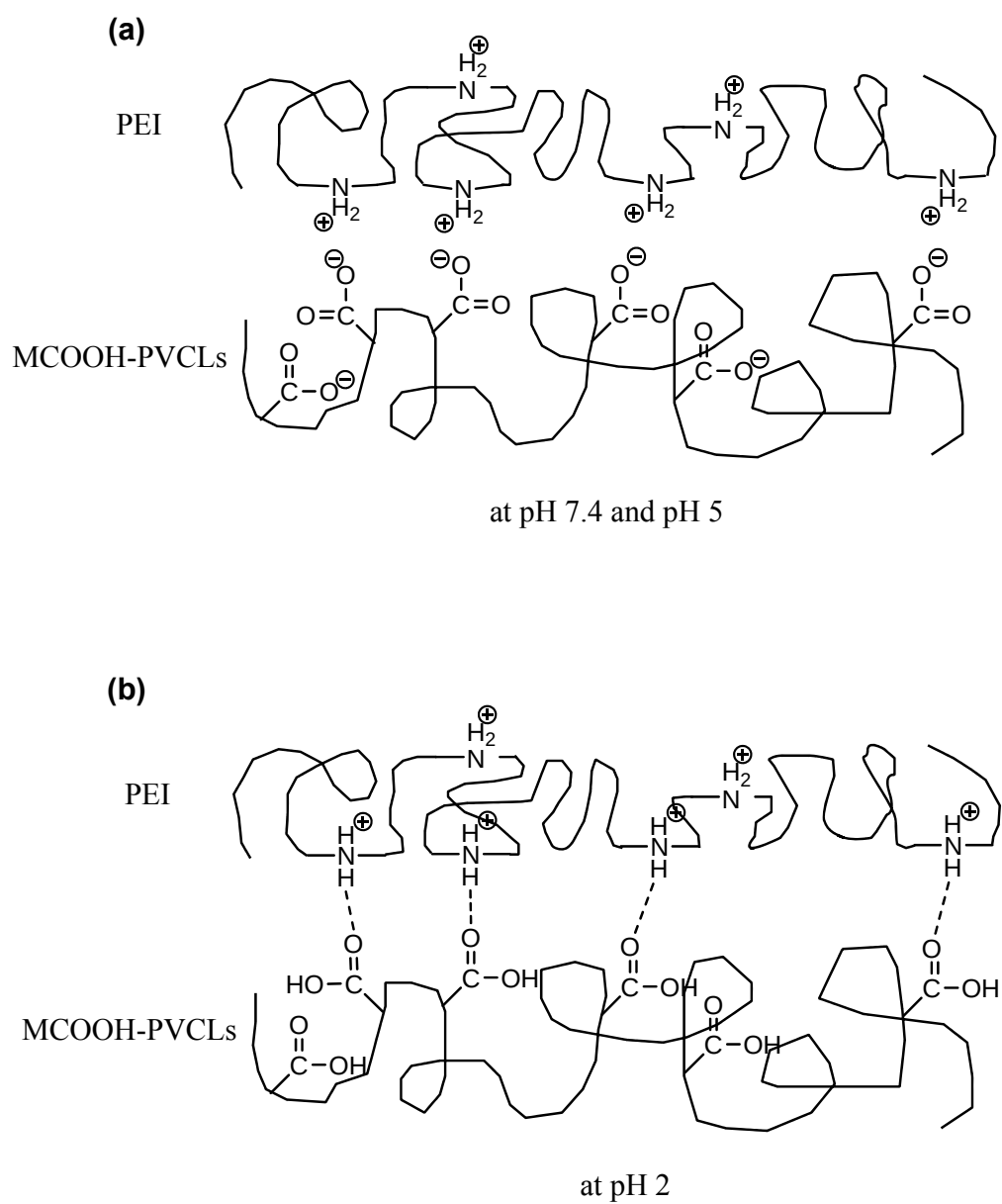


Figure 51 Schematics of bilayer of MCOOH-PVCL 19 and PEI (a) at pH 7.4 and pH 5, and (b) at pH 2.

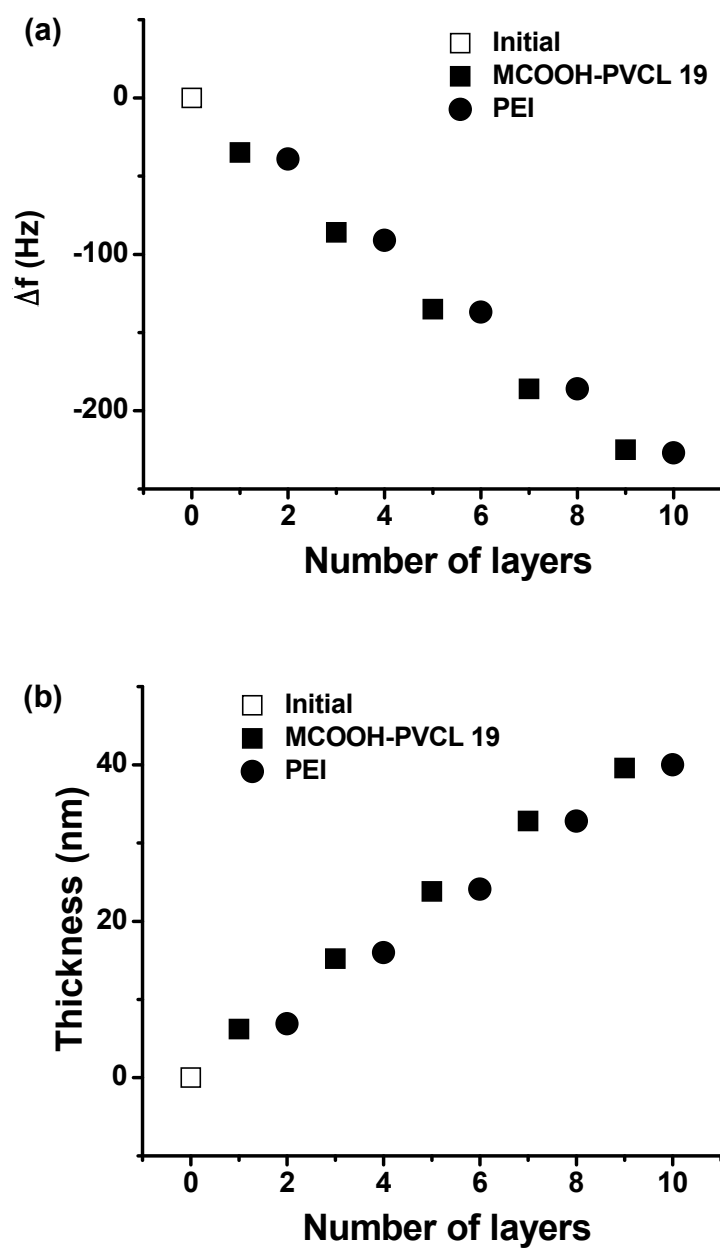


Figure 52 Layer-by-layer self-assembly using MCOOH-PVCL 19 and PEI in PBS at pH 7.4. The (a) Δf and (b) thickness were monitored using quartz crystal microbalance.

amount. It is reasonable because the PEI molecules possess much higher charge density than MCOOH-PVCL 19 per unit weight. The PEI exhibited a high efficiency in deposition and adsorption via electrostatic interaction for the construction of multilayers with MCOOH-PVCL 19. Similar observation of LBL assembly of PEI and cellulose derivative was reported previously [149]. But no conclusion could be drawn because it is possible desorption of MCOOH-PVCL 19 occurred in the presence of PEI solution. The loss of materials with weak electrostatic attraction in LBL assembly with PEI was also observed using a dye for labelling [150]. For further study, the MCOOH- PVCL 19 could be labelled with dye moieties which could be detected after the incubation with the PEI solution.

For release of drug, the response behaviours of the multilayers toward pH and temperature are of great importance. The obtained multilayers were firstly treated with PBS at different pH. The pH responsiveness study was focused on acidic environment for two reasons. First, the pathological tissues in vivo commonly present low pH ranging from 5.8 to 7.0 [151]. Second, the multilayers might deconstruct at high pH considering the pK_b of PEI being 8.8, above which the PEI would deprotonate and the electrostatic interaction between layers would be disrupted. As shown in Figure 53, the multilayers showed slight decrease in thickness but no drastic change in response toward the decrease of pH in the flowing PBS. The decrease of thickness is expected because the solubility of MCOOH-PVCL 19 decreases as pH decreases due to the protonation of the carboxylic acid group [76], resulting in the exclusion of water. The magnitude of the decrease was smaller than expected because the protonation of MCOOH-PVCL 19 caused the multilayers to become loosely structured, due to weakened electrostatic interaction. When

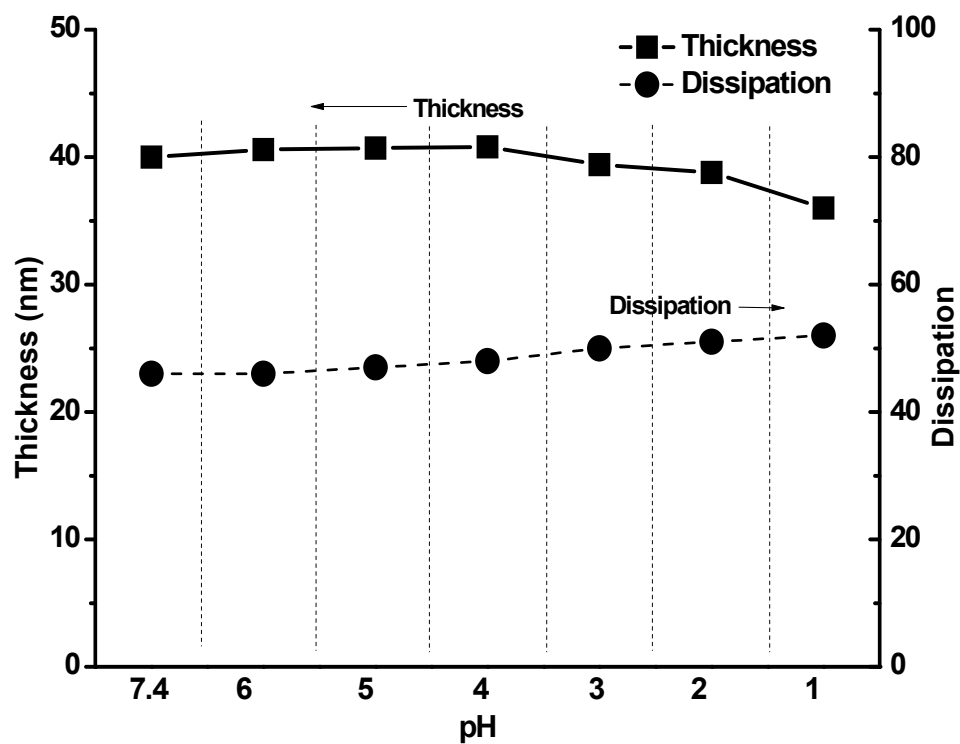


Figure 53 The thickness and dissipation of the (MCOOH-PVCL 19/PEI)×5 multilayers in response to pH.

the pH decreased below the pK_a of MCOOH-PVCL 19 at 3.1, the destruction of the multilayers and resulting drastic decrease in thickness were expected considering the completely loss of electrostatic interaction. However, no destruction was observed when the pH decreased below 3 (Figure 51). It can be explained that the hydrogen bonds formed between the hydrogen of amino group and the oxygen of carboxylic acid group at a weak level maintained the structure of the multilayers.

The temperature responsiveness was investigated as well. Because the copolymer MCOOH-PVCL 19 is dual responsive toward temperature and pH, three pH values were selected for the temperature study, including pH 7.4, pH 5, and pH 2. At physiological pH of 7.4, the multilayers showed reversible temperature responsiveness in a clear decrease in thickness from 40 nm to 27 nm when the temperature was raised from 22 °C to 37 °C (Figure 54a). The dissipation decrease (about 50%) as temperature increase indicated a corresponding result that the contraction of the layers along with increase of rigidity [152]. It is interesting to notice that at pH 5 near pathological pH the multilayers responded in a similar behaviour with that at pH 7.4 (Figure 54b). Because the cloud point of MCOOH-PVCL 19 at pH is around 32 °C, drastic response is expected considering the solubility of the copolymer changed dramatically. The reason could be that the restricted molecular rearrangement by compacted layers prevented copolymer molecules from adopting conformations [153]. The temperature responsiveness at pH 2 appeared very similar with those at pH 5 and pH 7.4 (Figure 54c) as well. At pH 2, the electrostatic interaction was replaced by weak force of hydrogen bond. However, the hydrogen bond could still keep the structure of the multilayers and inhibit the conformation adopting of copolymer chains as well.

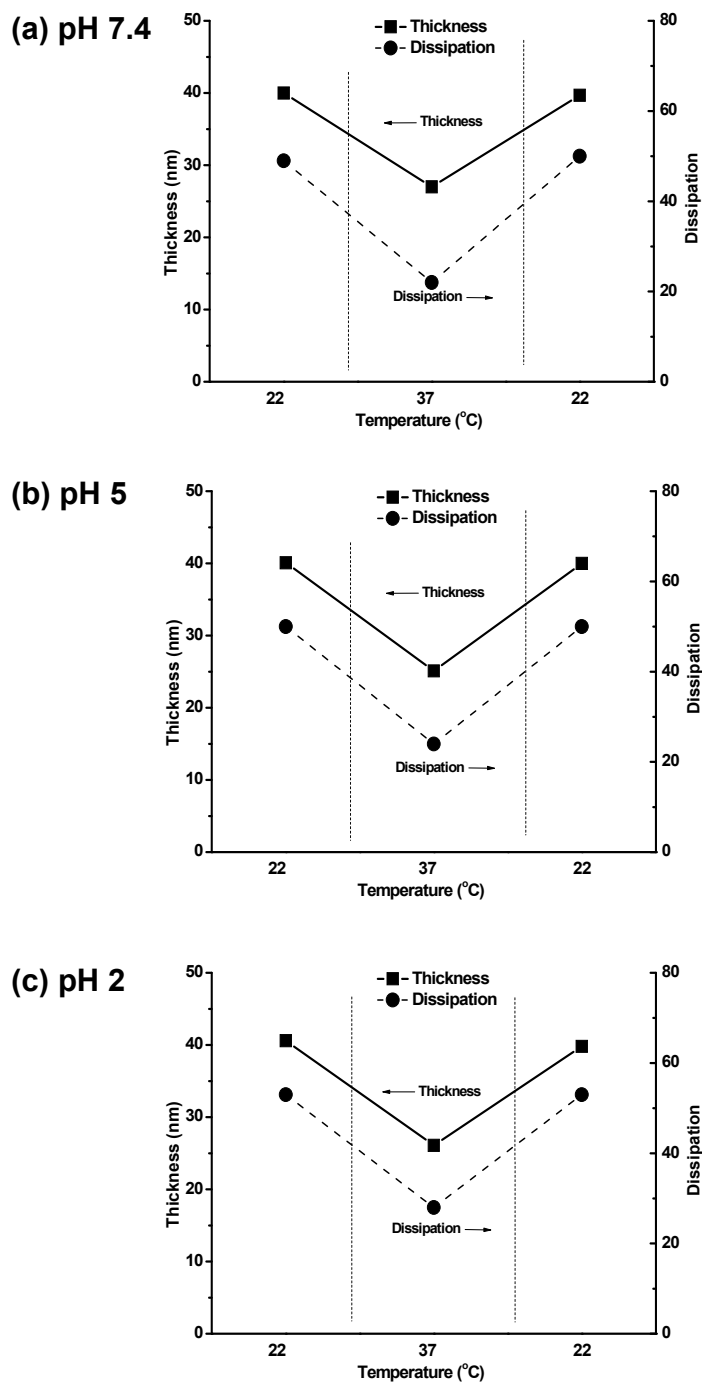


Figure 54 The thickness and dissipation of the (MCOOH-PVCL 19/PEI)×5 multilayers in response to temperature at (a) pH 7.4, (b) pH 5, and (c) pH 2.

3.4 Conclusions

A new functional derivative of the monomer *N*-vinyl-2-caprolactam (VCL) was designed to bear a *tert*-butoxycarbonylmethyl group at the 3-position of VCL. The monomer (TBMVCL) was successfully synthesized via nucleophilic substitution facilitated by a lithium enolate intermediate. The TBMVCL monomer allowed homologous copolymerization with VCL to obtain functional copolymers of VCL. The degree of functionality was easily controlled by varying the monomer feeding ratios. Similar to homopolymers of VCL, the copolymers were temperature responsive and an inverse correlation was observed between cloud points of the copolymers and their compositions. Furthermore, the deprotected copolymers exhibited pH-dependent thermoresponsive behaviors, due to protonation-deprotonation of the carboxyl groups at various pH and the associated change of polymer hydrophilicity. It was also found that the chemical nature of the carboxyl substitution group (i.e. number of carbons therein) can affect the cloud points as a function of pH. The effect is likely correlated to both hydrophobicity and size of the substitution group. The acute cytotoxicity assay of the deprotected copolymers exhibited great biocompatibility at low concentration. The successful self-assembly via layer-by-layer with a cationic polymer proves the potential application as a dual-responsive electrolyte for surface modification. The clear responsiveness of the obtained multilayers toward temperature was confirmed. Future study in effects of additional hydrophobic aliphatic groups between the caprolactam ring and carboxylic acid group to the dual-responsive behavior will aid in the design of new VCL derivatives.

Chapter 4 Preparation and Characterization of Multi-responsive Macro-porous Functional Hydrogel Scaffold for Neuronal Regeneration

4.1 Introduction

Hydrogels define a category of three-dimensional (3D) crosslinked polymeric gels with high water content [154]. The 3D network of hydrogels could provide a platform for cell function, adhesion and transplantation, and allow effective immobilization and sustained release of signaling biomolecules. The porous structure would benefit the diffusion and transportation of gas, nutrients and water-soluble metabolites. Therefore increasing attention has been paid to the biomedical applications of hydrogels, including cell-based therapeutics, soft tissue engineering, and drug delivery, etc [155].

Natural or synthetic, linear or branched polymers can be physically or chemically cross-linked for fabrication of hydrogels [156]. The physical gels are commonly formed via the ionic interaction, hydrogen bonding, and hydrophobic forces [157]. The hydrogels with covalent bonds are usually prepared in two different approaches: direct polymerization of monomers with multi-functional groups in order to form 3D structure, or crosslinking of presynthesized polymers or oligomers [158]. Direct polymerization, e.g. free-radical polymerization, can be induced using initiators which are sensitive to thermal [159] or light (UV) stimuli [160], as well as directly triggered by other energy resources such as gamma- [161] or microwave-radiation [162]. However, the purification of residual monomers via rinsing is diffusion-dependent and time consuming [163]. In order to reduce the amount of small molecule residual, the presynthesized polymers and

oligomers are introduced for hydrogel preparation. These oligomers can be designed and synthesized for incorporation of desirable properties into the hydrogel, such as biodegradability [164].

The polymeric hydrogels as scaffolds are favorable for peripheral and central nerve regeneration [165] considering their tunable stiffness, variable functional moieties, and drug loading capability [166]. The scaffolds reported are mainly in two forms including three-dimensional meshed matrix [167] and tubes [168] with different types of polymers such as hyaluronic acid [169], poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) [170], and poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) [171], etc.

The mesh size of the scaffold is critical to the migration of neurons and outgrowth of neurites. The size of neurons cell body is 20~30 μm while the diameter of the axon is about 1 μm [172]. The pore size larger than 30 μm would benefit the penetration of neural cells into the scaffold as well as the neurite extension [173].

There is growing interest in integrating additional characteristics into hydrogels to expand their functional roles [174]. In the context of neural regeneration, it is desirable to have hydrogels not only support neuron adhesion and neurite extension, but also capable of neural protection against hostile pathological surroundings. Herein we present a new system that bears the aforementioned features.

In this hydrogel, the functional groups can be protonated at physiological conditions, thus rendering a cationic nature to the hydrogels to promote adhesion of neurons with negatively charged surface and potentially allow for delivery of growth factors via electrostatic binding effects. Additionally, macro-porosity and interconnectivity would significantly benefit the neural cells penetration, nutrient

diffusion, and tissue metabolism [175]. Furthermore, a particulate microstructure of scaffold can potentially serve as reservoirs to hold and deliver therapeutics locally to increase cell survivability.

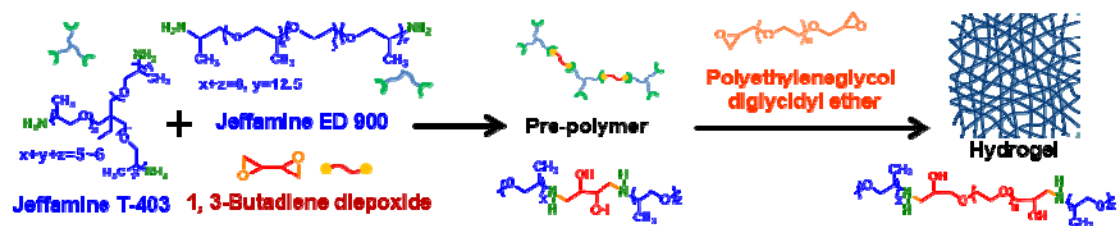
In this chapter a degradable macro-porous multi-sensitive hydrogel preparation via an amine-epoxy ring opening reaction is presented. A pre-polymer is first obtained via polymerization of Jeffamine T-403 (JT403) and 1, 3-butadiene diepoxide (Scheme 6). The lower critical solution temperature (LCST) of the pre-synthesized polyetheramine polymer is between room temperature and 65 °C. Then the pre-polymer is mixed with polyethyleneglycol diglycidyl ether (PEGDE) in aqueous solution. In the reaction at 65 °C, the two reactants crosslink and form a 3D network while phase separation occurs and leads to hierarchical micro-scale interconnect porous structure.

In addition, Jeffamine ED-900 (JED900) was introduced as a second Jeffamine into this hydrogel system to vary the crosslink density. As a linear oligomer with two amino-terminal groups, JED900 is different from the branched molecule JT403 with three amine moieties. Thus the obtained hydrogel is of lower crosslink density considering the less amount of functional groups for network formation.

4.2 Experimental Section

4.2.1 Materials

Jeffamine T-403 polyetheramine (JT403) and Jeffamine ED-900 polyetheramine (JED900) were kindly provided by Huntsman Chemical. 1, 3-Butadiene diepoxide (BDE), polyethyleneglycol diglycidyl ether ($M_n=500$) (PEGDE), and polyethylenimine (PEI, branched, $M_n = 20,000$ g/mol) were purchased from Sigma-Aldrich. Hydrogen peroxide



Scheme 6 Synthesis route of macro-porous hydrogels via polyetheramine pre-polymer.

(30%) was obtained from Fisher Scientific. Nile red was kindly provided by Dr. Bin Zhao. Fluorescein isothiocyanate isomer I (FITC) was purchased from Acros Organics (90%, pure). All chemicals were used as received.

4.2.2 Prepolymer preparation and characterization

The hydrogel prepolymer solution was prepared by dissolving JT403 in water before adding BDE for a homogeneous mixture. The ratio between amino hydrogen and epoxide groups was kept at 3:1, while the total concentration of the reactants was 15 wt%. For the TED hydrogel prepolymers, JED900 was added while the amino hydrogen ratio of JT403 to JED900 was 2:1. The resultant solution was then allowed to react in a constant temperature water bath at 65 °C for 30 minutes after which it was immediately cooled to room temperature by placing the vial under cold water and vortexing until homogeneous.

The lower critical solution temperature of the prepolymer was studied by the Thermoscientific Evolution 600 UV-VIS spectrometer. The prepolymer was dissolved at a concentration of 2.5 mg/mL in DI water. The solution was heated up at the step of 1 °C with an equilibration of 2 min for each temperature.

In order to characterize the molecular weight, the prepolymer was dissolved in DI water at a concentration of 0.3 mg/mL with 0.02% of NaN₃. The solution was then analyzed using an aqueous gel permeation chromatography (GPC). The GPC has a Waters model R401 differential refractometer and four columns: Polymer Standards Service suprema; 8x300 mm; 10 µm; 100, 3000, and 10,000 Å as well as a 8x50, 10 µm guard. The instrument was calibrated from a molecular weight range of 600 to 1,000,000

Daltons using PEO/PEG standards. All data were collected and analyzed using Polymer Labs Cirrus software.

4.2.3 Preparation of the hydrogel

For the T hydrogel, 200 mg of PEGDE (158 mg for the TED hydrogel) was added to a 1 mL prepolymer solution and mixed until homogeneous. The mixed solution was placed into a rounded polydimethylsiloxane (PDMS) mold with a diameter of 25 mm. To prevent leaking or seepage of solution, the PDMS molds were sealed to a PTFE sheet using a light, even coating of high temperature vacuum grease. The molds, sheet, and mixture was then enclosed in a customized polypropylene container half-filled with water to maintain a humid environment throughout the reaction and allowed to react in an oven at 65 °C for 1 hour. For swelling tests, samples were prepared in a 17 mm diameter capped vial under the same reaction conditions. The hydrogels were rinsed 5 times with DI water for 10 hours incubation at each time before further characterization. The micro-morphology of the skin layer of the hydrogel were different from the inner part because of the irregular phase separation in a restricted space near the inside wall of the container. So the skin layer were removed to keep the uniformity of the samples.

4.2.4 Characterization

4.2.4.1 Swelling behavior

The samples were punched to pie shape with diameter of 12 mm using a biopsy punch. The hydrogels were freeze-dried after which the dehydrated mass and diameter were measured.

The dried samples were then placed in the 0.1 M phosphate buffered saline (PBS) solution and allowed to swell at 37 °C. Measurements of diameter and mass were taken at

10 minutes, 30 minutes, 1 hour, 2 hours, and 24 hours in order to determine swelling rate and equilibrium swelling conditions.

At the designated time point each sample was blotted using filter paper for 1 second per side to remove excessive PBS solution, after which the samples were massed and diameter measured with a vernier caliper. Experiments were repeated three times.

The normalized weight was determined via the following equation:

$$Normalized_weight = \frac{m_s}{m_i} \quad (1)$$

where m_s is the swollen mass and m_i is the dried (or initial) mass of the hydrogels.

The normalized volume was determined via the following equation:

$$Normalized_volume = \left(\frac{d_s}{d_i} \right)^3 \quad (2)$$

where d_s is the swollen diameter and d_i is the dried (or initial) diameter of the hydrogels. Gravimetric and Volumetric swelling ratios were then calculated based upon Equation 1 and 2, respectively.

4.2.4.2 Rheological measurements

The rheological properties of the hydrogels were obtained by a stress-controlled rheometer (TA Instruments Model TA AR2000ex). The hydrogels were cut into 20 mm diameter samples for the cone-plate geometry at a cone angle of 2° (truncation 52 µm); the loading force was adjusted and kept to 3 N, and the temperature was controlled by the bottom Peltier plate. The frequency sweep tests from 0.1 to 100 Hz were conducted to study the frequency dependencies of G' and G'' . The hydrogel was equilibrated for 3 min prior to data recording at each temperature. A strain amplitude was set as $\gamma = 1\%$ in all the

tests.

4.2.4.3 Scanning electron microscopy

The hydrogels were freeze-dried, mounted on aluminum studs, and sputter-coated with gold, and observed by a LEO 1525 scanning electron microscope. The working distance was kept around 6 mm while the beam voltage was 3 kV.

4.2.4.4 Confocal observation

The hydrogels were stained with Nile red and FITC dyes respectively.

For Nile red loading, the acetone solution of Nile red (1 mg/mL) was diluted in water at 65°C at the volume ratio of 1:10. The hydrogels were immersed in the aforementioned solution at 65 °C for 20 min and then cooled down with ice bath. After that, the hydrogels were kept in the mixture at 4 °C overnight before being rinsed with DI water for 3 times.

For FITC labelling, the FITC powder was dissolved in acetone at a concentration of 1 mg/mL. Then 0.5 mL FITC solution was mixed with 3 pieces of the hydrogels (5 mm X 5 mm X 10 mm) in 4 mL aqueous solution. After overnight incubation at 4 °C, the hydrogels were rinsed with DI water for 3 times.

The Nile red loaded or FITC labeled hydrogels were then sectioned into thin pieces and observed under FluoView FV1000 Confocal microscope (Olympus). A series of z-stack confocal microscopy images were taken using the 60X object lens to generate a representative image of the hydrogel. The step width was 2 µm to collect details of the hydrogels considering the dimension of their micro-structure.

4.2.4.5 Degradation behavior

The degradation behavior of the T hydrogels was evaluated using hydrogen peroxide which is a common oxidizing agent in the neural tissue. The hydrogel disks with diameter of 6 mm were prepared using 25 mm hydrogel disks and a 6 mm biopsy punch. The samples were rinsed to remove debris and immersed in 30 mL of 5% H₂O₂ solution. At each 24 h interval the hydrogel was removed and gently rinsed with DI water before it was massed and had its diameter measured. For the TED hydrogels, the time interval was 2 h. Measurements were continued until the sample was no longer physically stable enough to measure. Gravimetric and volumetric swelling ratios were calculated based on Equations 1 and 2, respectively.

Characterization of the hydrogel microstructure during degradation was performed using a LEO 1525 scanning electron microscope. At the designated time point the hydrogel was removed, gently rinsed, freeze-dried, sputter-coated with gold, and observed. The working distance was kept around 6 mm while the beam voltage was 3 kV.

4.2.5 In vitro cytotoxicity study

The acute cytotoxicity of the hydrogels was determined via direct contact culture with NIH/3T3 fibroblast following ISO standards [176]. The fibroblasts were seeded to 24 or 48 well plate at density of 7.8×10^4 cells/cm² and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a 5% CO₂ atmosphere for 16 h. The cell confluency reached about 80% at the beginning of treatment. For optical images, each sample was cut into 5 mm × 5 mm square and incubated with the cells in a well of 24 well plate in 400 μL culture medium.

Before the treatment, all samples were sterilized by 30 min UV exposure and

equilibrated in culture medium for 12 h at 37 °C. At the beginning of the treatment, the culture medium was replaced by fresh culture medium before individual samples being carefully placed on the cell layer in the center of each well. After incubation at 37 °C for 24 h, cell culture was examined microscopically using a Zeiss Observer A1 inverted optical microscope for cellular response.

For qualitative study, the samples (0.25 cm²) were individually kept in contact with the cells in the 24 well plate (1.9 cm² per well). For quantitative study, the samples (0.096 cm²) were individually kept in contact with the cells in the 48 well plate (0.95 cm² per well). At the end of the treatment, hydrogel samples were removed followed by replacing the cell culture medium with 300 µL WST solution (WST-1 reagent to medium 1:10 in volume). The absorbance at 440 nm was obtained after 2 h incubation at 37 °C using a BioTek Synergy 2 Multi-Mode Microplate Reader (BioTek, USA) against a blank control for background correction.

4.2.6 Primary cortical neuron culture

Cortical neurons were obtained from 7-day-old chicken embryo. Forebrains of the embryo were dissected, minced into small pieces, and enzymatically dissociated with 0.25% trypsin in PBS for 20 min at 37 °C, followed by inactivation with medium containing 10% fetal bovine serum (FBS, Invitrogen). A cell pellet was obtained after a brief centrifugation, and mechanical trituration using a fire-polished Pasteur pipet was applied to further dissociate the cells. It has been reported that this process provides the culture with a predominantly neuron composition [177]. Neuronal cells were collected and resuspended for cell culture.

Cortical neurons were seeded onto the cut exposed-interior of the hydrogel at a

density of 100,000 cells/cm² and cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine at 37 °C in a 5% CO₂ atmosphere for treatment. The hydrogels were cut into 5 mm × 5 mm × 2 mm pieces and incubated in a 35 mm petri dish before neuron cells being transferred into the dish. The treatment period were 4 h, 24 h, 48 h, and 120 h for the T hydrogel, and 24 h, 48 h, and 72 h for the TED hydrogel respectively. Cell viability was investigated by comparing the number of live calcien AM stained cells on the hydrogel with cells seeded on a Poly-L-Lysine coated 35 mm dish for control. Fluorescent staining of the cells was done by first rinsing the samples 2x with sterile PBS to remove any unattached cells, submerging the sample in PBS solution with calcien AM at the concentration of 2 μM for 20 min at 37 °C, finally replacing the staining solution with PBS before imaging.

The SEM samples were prepared via fixation and gold coating following previous protocol [55]. Briefly, samples were rinsed twice with PBS before being fixed using 4 % glutaldehyde in PBS for 24 h at 4 °C. After being rinsed with PBS for three times, samples were dehydrated via 10 min incubation each in ascending grades of ethanol baths (25, 50, 75, and 100%). After that an additional dehydration was performed using dehydrated ethanol for 10 min incubation. Hexamethyldisilazane (HMDS) was used for further dehydration to warrant a thorough removal of water. Samples were incubated in the mixtures of dehydrated ethanol and HMDS at a series of ratios at 2:1, 1:1, 1:2, and 0:1 for 10 min each. Samples were then preserved under house vacuum to allow a complete evaporation of HMDS. Dehydrated samples were then coated with gold using an SPI sputtering device for 20 s at 20 mA for SEM imaging.

4.3 Results and Discussions

4.3.1 Prepolymer synthesis and characterization

The T hydrogel prepolymer synthesized from JT403 and BDE was used without further purification. In addition to the JT403 based prepolymer of the T hydrogel, JED900 was incorporated in the synthesis of the TED hydrogel prepolymer. The JED900 contains 12 ethylene oxide repeating units instead of 5~6 propylene oxide units in JT403. So larger size and higher hydrophilicity of TED prepolymer were expected than those of T prepolymer.

Here in both prepolymers the ratio of functional groups between amino hydrogen and epoxide was kept at 3:1. In the T prepolymer all the amino hydrogen was introduced from JT403, so the molar ratio of reactants JT403 to BDE was 1:1. In the TED prepolymer the ratio of amino hydrogen between JT403 and JED900 was 2:1, while the molar ratio of reactants JT403 to JED900 was 4:3. Therefore the molar ratio of JT403: JED900: BDE was 4:3:6.

The number average molecular weight (M_n) of the T hydrogel prepolymer was determined to be around 1,400 Da using water GPC with lyophilized samples. Generally, one prepolymer molecule could be composed of three 400 Da JT403 molecules. The molecular weight of the TED hydrogel prepolymer was not detected because the water GPC curve showed no peak of refraction in the calibration range of retention time. The TED prepolymer is speculated to be larger than T prepolymer considering 900 Da JED900 in the composition. Then the large molecular size might attribute to the fast depletion from the column in a short elution time in GPC resulting an apparent no peak in the curve.

Solution behavior of the polymers could be also used for qualitatively comparison of the molecular weights, referring to the relationship between the LCST and Mw. The pure JT403 molecule weight is relatively small showing a phase transition point higher than the reaction temperature 65 °C. As the polymerization occurs, the molecule weight increases along with the growth of polymer chains, resulting decrease of cloud point. It was confirmed in Figure 55a that the LCST of the T prepolymer is 28 °C. The TED prepolymer demonstrated a gradual increase of cloudiness but no sharp transition below 75 °C. Furthermore, the size of coacervates in the prepolymer solution was monitored using zetasizer as the temperature being raised (Figure 55b). For the T prepolymer, the hydrodynamic size was constant at 10 nm when the temperature increased from 0 °C to 25 °C. Between 25 °C and 30 °C, drastic increase of size from 10 nm to 1200 nm was observed followed by a clear decrease in size from 1200 nm to 900 nm as temperature raised from 30 °C to 45 °C. The T prepolymers are soluble and well dispersed below the LCST (28 °C), showing a fine hydrodynamic size (10 nm). Between 25°C and 30 °C, corresponding with the LCST at 28 °C, the T prepolymers formed coacervates containing folded chains because the solvent was no longer favorable. The degree of immiscibility between polymer and water molecules increased as temperature increased, leading to the decrease of hydrodynamic size. The change of prepolymer hydrodynamic sizes in response to temperature were able to support the explanation of the inner structure and morphology of the obtained T hydrogel. At the given reaction temperature (65 °C) higher than transition point (28 °C), phase separation occurred while the polymer molecules formed coacervates. These coacervates were later intra-

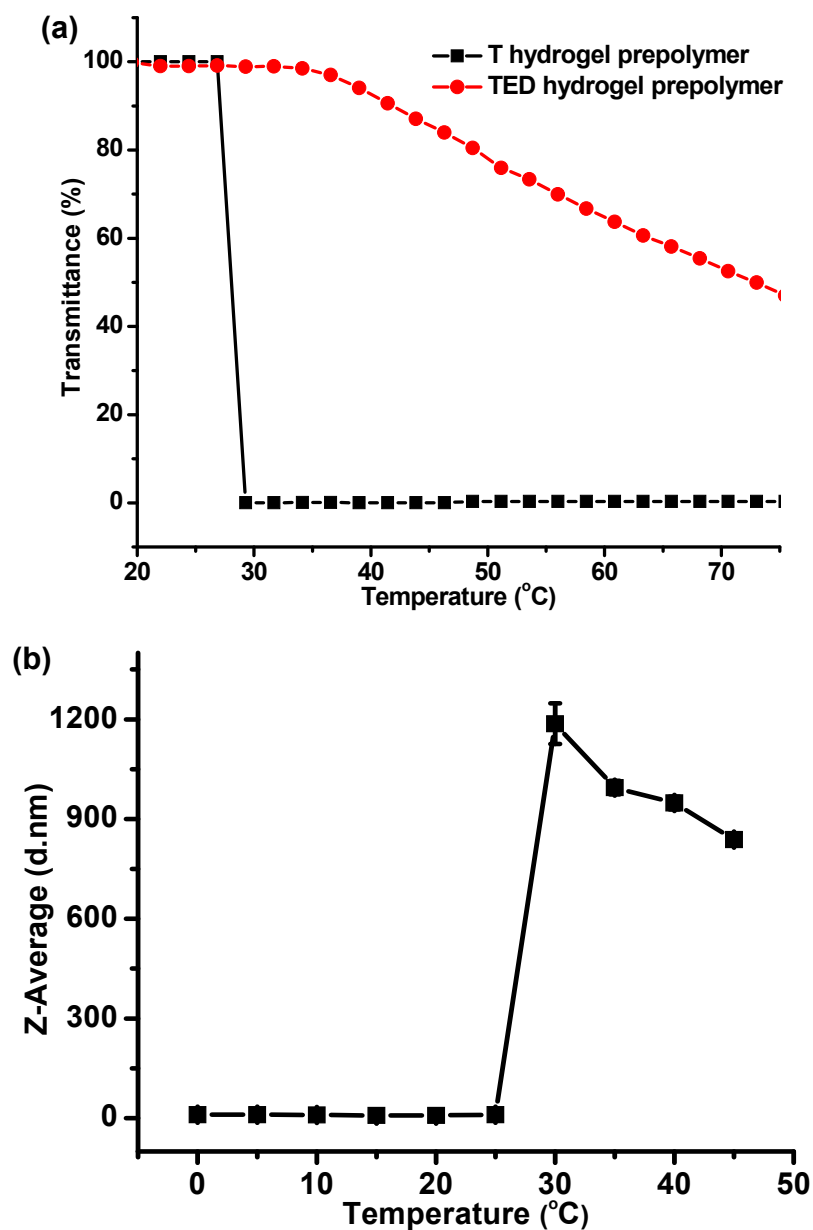


Figure 55 (a) Phase transition profile of the T hydrogel prepolymer and transmittance of the TED hydrogel prepolymer solution as a function of temperature. The concentration of prepolymer was 2.5 mg/mL. (b) Hydrodynamic sizes of the T hydrogel prepolymer as a function of temperature.

crosslinked and inter-connected via coupling agent PEG diepoxide. For the TED prepolymer, no hydrodynamic size was detected using zetasizer so no data was obtained. It is probable that the good affinity of the TED prepolymer with water due to the additional JED900 resulted in lack of laser diffraction for detection.

4.3.2 Characterization of macro-porous hydrogel

4.3.2.1 Morphology of macro-porous hydrogels

Cationic macroporous hydrogels were prepared via ring-opening reaction of polyetheramine prepolymers and PEG-diepoxide. For the hydrogel preparation, two compositions of reactants were applied referring to previous investigation of polyetheramine based gel particles [67, 74], including the T hydrogel and the TED hydrogel. In the aqueous mixture for the T hydrogel, phase separation of prepolymer occurred during incubation and subsequently induced hierarchical structure at micro-scale. In the TED hydrogel preparation, no obvious evidence for phase separation was observed.

The visual appearance of the T and TED hydrogels were obtained using a digital camera. As it was mentioned previously, JED900 is a linear oligomer with two amino-terminal groups, while JT403 is a branched molecule with three amine moieties. Thus the TED hydrogel is of lower crosslink density considering the less amount of functional groups for network formation. It is expected the TED hydrogel would show higher transparency than T hydrogel because of the lower crosslink density and resulting lower level of difference in refractive index of sample from the surrounding water. The expectation was supported by the comparison in optical observation of the two samples (Figure 56a-b). Comparing samples of the same dimension, the T hydrogel was opaque

(a) The T hydrogel visual observation



(b) The TED hydrogel visual observation



Figure 56 Visual observation of (a) T hydrogel and (b) TED hydrogel.

and white while the TED hydrogel was semi-transparent.

In order to maintain the morphology of micro-structure, the hydrogels were lyophilized for SEM imaging. The T hydrogel shrank slightly but its structure was kept as shown in Figure 57. However, the TED hydrogel collapsed during the drying process to a thin layer of dehydrated film (Figure 58). The SEM image of dehydrated T hydrogel showed that the scaffold contained interconnected solid-like spherical particles with diameter around 5 μm . These particles attached to each other randomly forming an interconnected cavity homogeneously distributed in the scaffold. No small pore was observed on the surface of these spherical particles in SEM images, likely due to the shrinking during dehydration. As aforementioned in the section of prepolymer characterization, the phase separation of T prepolymer occurred during the course of hydrogel formation, so the morphology and dimension of the coacervates would affect the hydrogel structure. The hydrodynamic size of T prepolymer between 30 °C and 45 °C suggested a decrease trend as a function of temperature. So it can be expected the size of T prepolymer coacervates would be sub-micron at 65 °C. However, the structure of the obtained T hydrogel exhibited spherical particles at the micron-scale. It is reasonable considering the growth of chain length because of the reaction with the coupling reagent PEGDE and the resulting viscosity increase of the mixture. For the TED hydrogel (Figure 58), the wrinkle-like surface seems like deposition of several soft layers. The dehydration process resulted in a drastic collapse of 8 mm thick scaffold of the TED hydrogel while limited information about morphology of hydrated hydrogel can be obtained from the SEM sample. Additionally, it was observed that small hard granules around 2 μm scattered in the matrix. These small spherical granules are similar to the particle structure

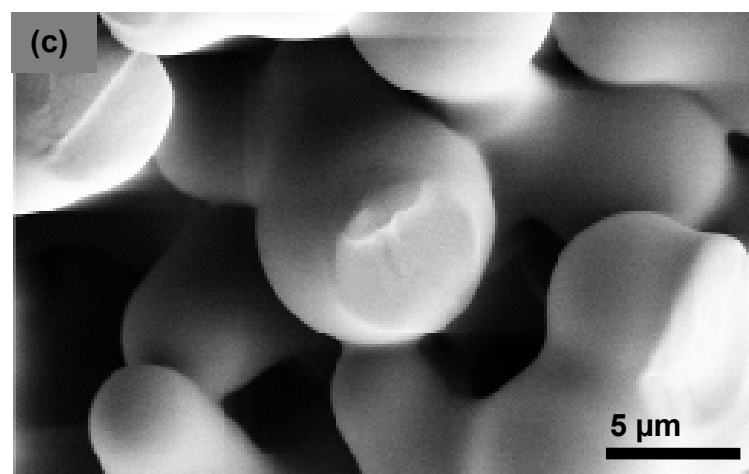
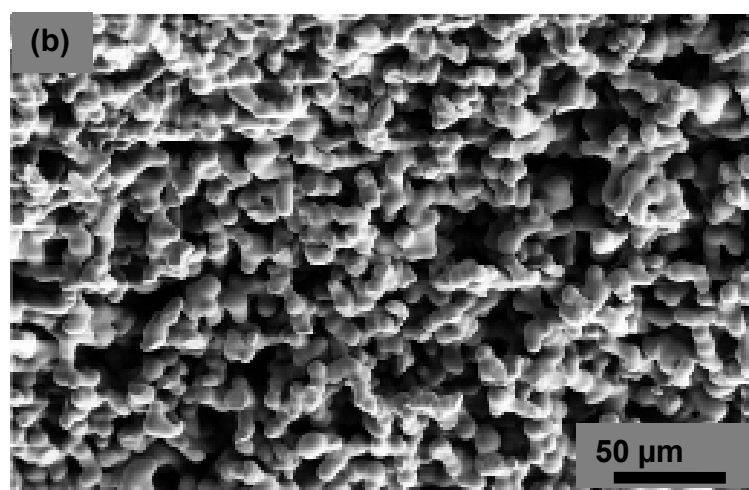
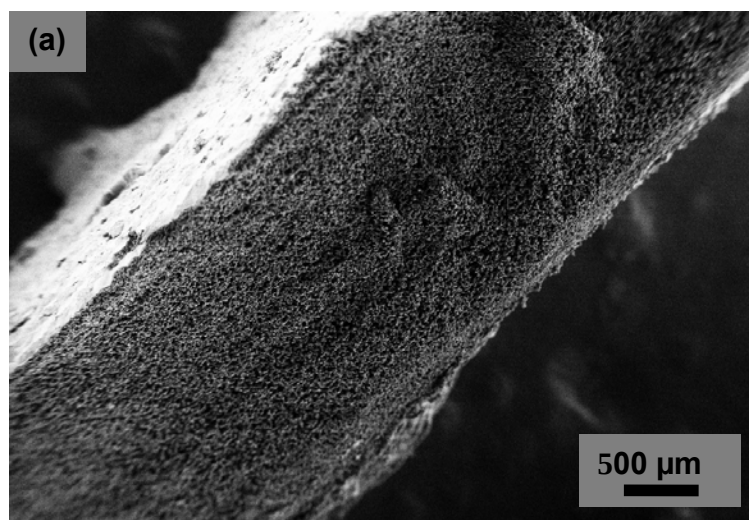


Figure 57 SEM images of lyophilized T hydrogel at different magnifications.

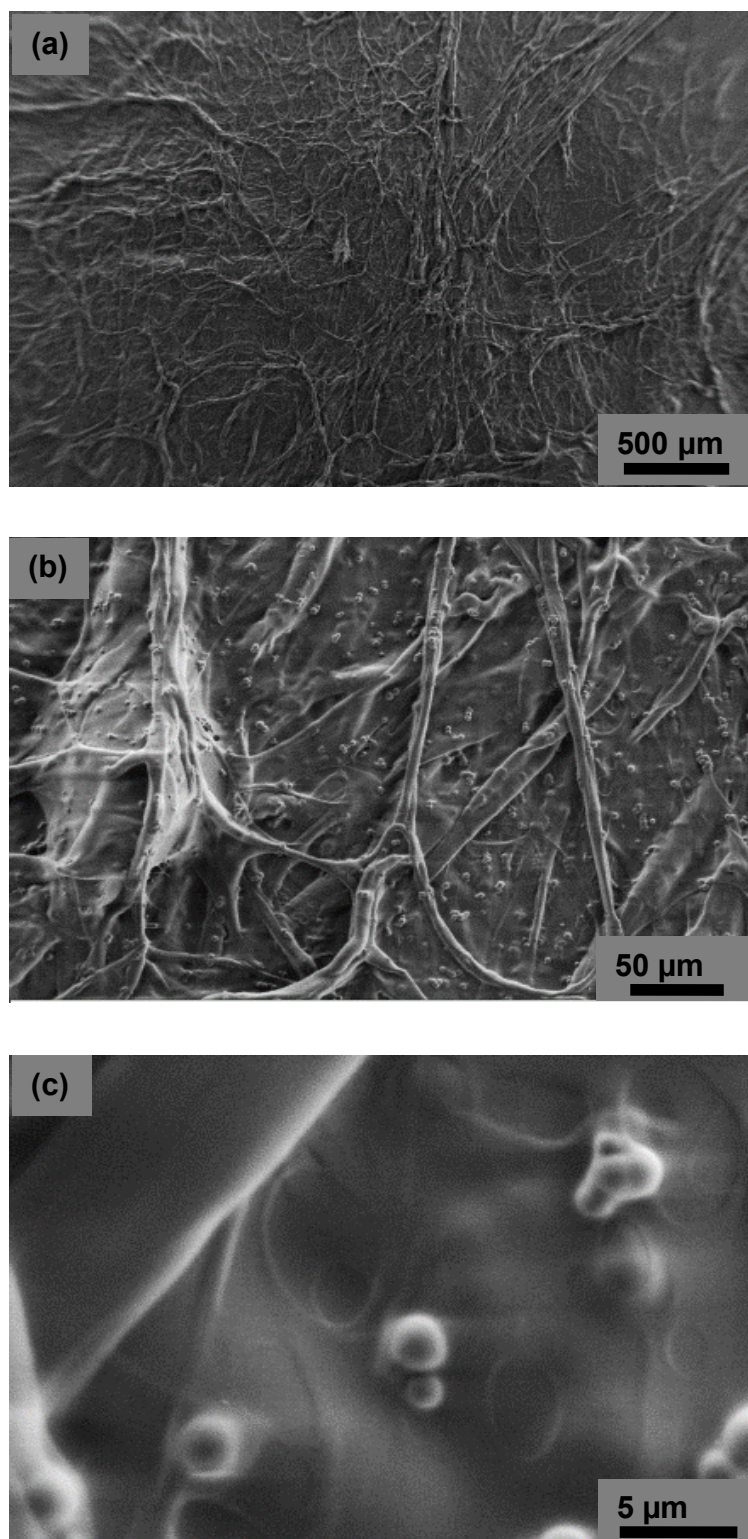
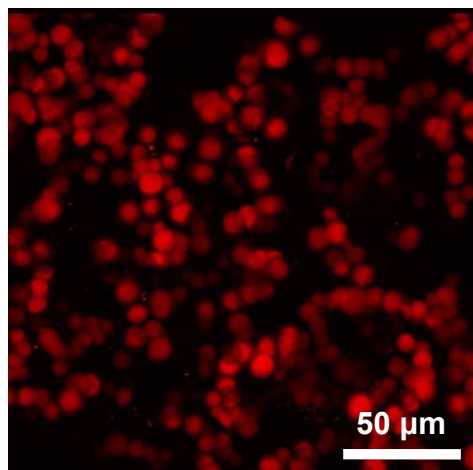


Figure 58 SEM images of lyophilized TED hydrogel at different magnifications.

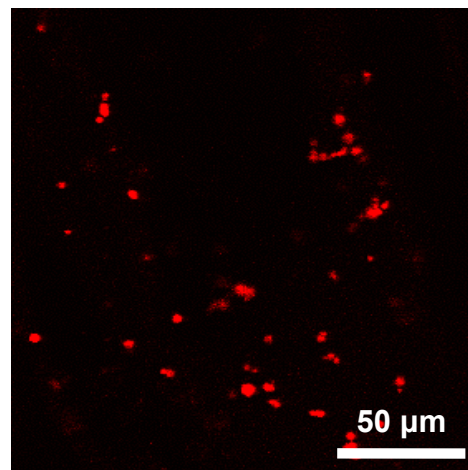
in the T hydrogel. It can be speculated that in the course of TED hydrogel formation, phase separation took place resulting only scattered individual granules due to the low concentration of the hydrophobic structure.

Such hydrogels are designed and fabricated targeting for biomedical applications under mild condition in a hydrated form, so it is of great importance to determine the morphology and micro-structure of hydrated hydrogels. The hydrogel samples were stained with Nile red and FITC respectively for fluorescent images (Figure 59). The Nile red was loaded to the hydrogels via hydrophobic interaction while the FITC reacted with the amino or hydroxyl groups of the hydrogel via the isothiocyanate group. The T hydrogels showed spherical domains in both Figure 59a and c, which are consistent with the observation in SEM image of the dehydrated samples. It suggested that the morphology was kept during the drying course. The Nile red stained T hydrogel showed individual spheres instead of interconnected structure observed in SEM image. The possible reason is that the necking part between adjacent spheres is hydrophilic considering the dominant role of hydrophobic interaction during loading of Nile red. Therefore, these hydrophilic areas without staining were invisible in the fluorescent image (Figure 59a). Additionally, a similar morphology was noticed in the fluorescent image of FITC labeled T hydrogel. It indicated that the spherical domains contained high amount of amine groups, which are likely introduced by the JT403, or hydroxyl groups obtained in the amine-epoxy reaction. The fluorescent images also demonstrated dimension and morphology of the frame and cavities of the hydrated hydrogels. The sizes of spherical domains are around 3~8 μm while the sizes of cavities ranged from 2 to 20 μm . For the TED hydrogel, a few scattered small domains labeled with Nile red were

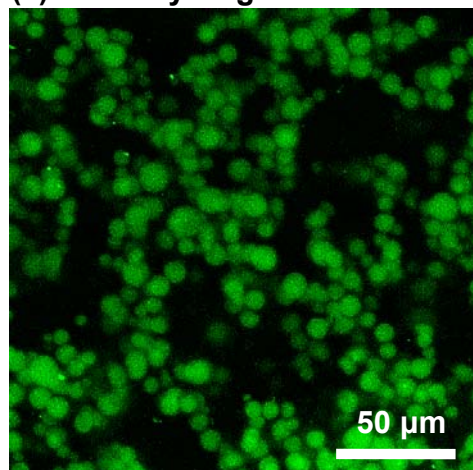
(a) The T hydrogel with Nile Red



(b) The TED hydrogel with Nile Red



(c) The T hydrogel with FITC



(d) The TED hydrogel with FITC

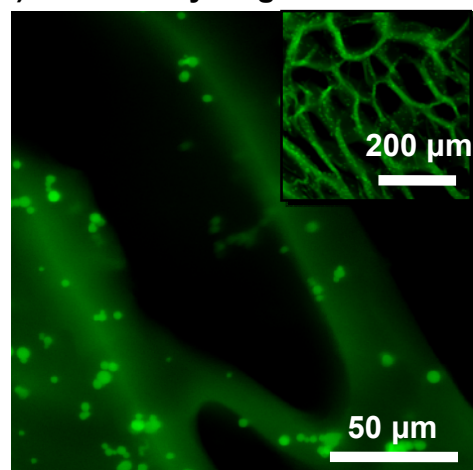


Figure 59 Confocal images of Nile red loaded T hydrogel (a) and TED hydrogel (b), and FITC labeled T hydrogel (c) and TED hydrogel (d).

observed. The small granules with diameters ranged 1~3 μm , which also appeared in the SEM images, are potentially applicable as reservoir for hydrophobic drugs considering their proven ability to encapsulate Nile red. The majority part of the scaffold did not show fluorescent intensity via Nile red loading because of hydrophilicity of the main scaffold due to the additional PEO structure from JED900. But the main structure appeared with the FITC labeling. The scaffold exhibited an interconnected network with branch thickness around 20~50 μm and an interconnected cavity whose channel diameters ranged from 30 μm to 50 μm . The hydrophilic JED900 units, which are not favored by hydrophobic interaction with Nile red, were marked with FITC via chemical reactions. This long-range order structure of the TED hydrogel (Figure 59d inset) offers potential in application of tissue engineering.

4.3.2.2 Swelling behavior of macro-porous hydrogels in response to temperature and pH

The swelling behaviors of the dehydrated hydrogels were performed in PBS at 37 $^{\circ}\text{C}$ to mimic the *in vivo* environment (Figure 60a). It was observed that the swelling became saturated around 30 min for the T hydrogel, and around 100 min for the TED hydrogel. For the T hydrogel, the gravimetric and volumetric ratios are 7.4 and 2.7 respectively. The TED hydrogel demonstrated an increase of 18 fold and 9.5 fold in mass and volume respectively. It is reasonable that the TED hydrogel showed a much higher swelling ratio than the T hydrogel because the relatively higher hydrophilicity and lower crosslink density allowed absorption of larger amount of water and less restriction in volumetric expansion.

The temperature responsiveness was determined via normalized the T hydrogel

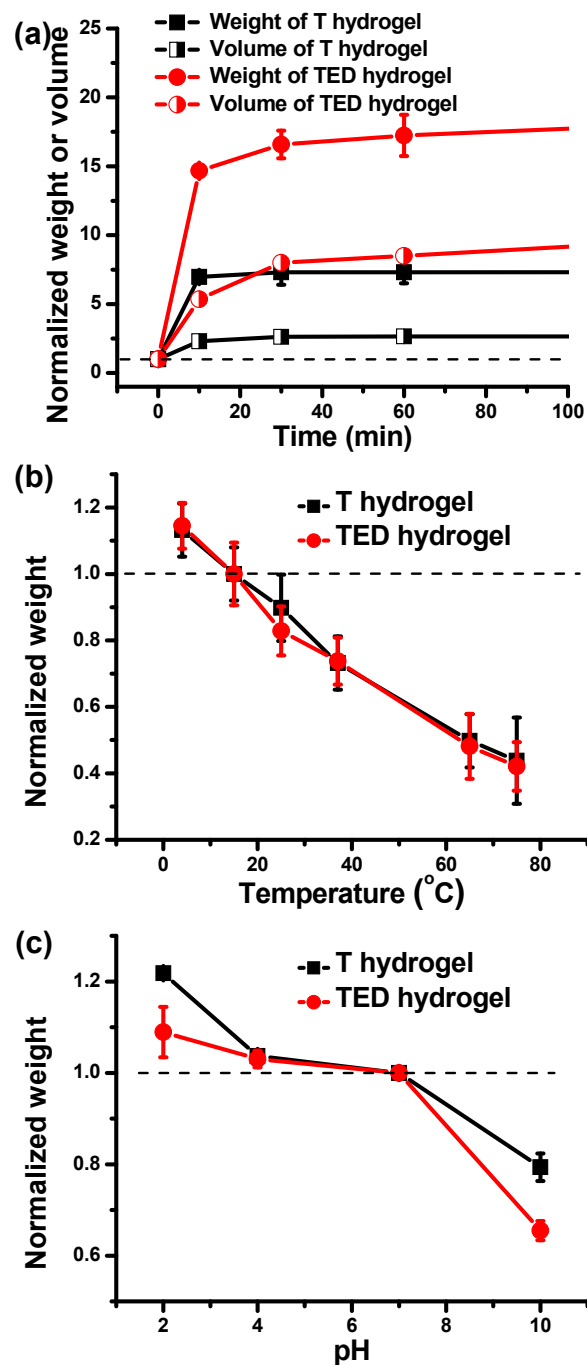


Figure 60 (a) Dynamic swelling of dehydrated hydrogels in PBS at 37 °C (normalized against dehydrated sample), (b) swelling behavior of hydrated hydrogels in PBS at pH 7.4 at different temperatures (normalized against 15 °C), (c) swelling behavior of hydrated hydrogels at different pH at room temperature (normalized against pH 7).

weights against the weight at 15 °C as well as the TED hydrogel (Figure 60b). The T hydrogel exhibited normalized weights of 1.13 at 4 °C, 0.90 at 25 °C, 0.73 at 37 °C, 0.50 at 65 °C, and 0.44 at 75 °C. The shrinking of the T hydrogel as temperature increases is consistent with the thermal response of T prepolymer. The TED hydrogel showed a similar behavior with the T hydrogel that the normalized weights of two hydrogels were very close at corresponding temperatures, such as 1.14 at 4 °C and 0.42 at 75 °C. It is interesting that the two hydrogels showed very close transition profile as a function of temperature despite the fact that TED hydrogel is more hydrophilic. Except for the normalized weight at 25 °C, the two swelling curves almost overlapped with each other. It is possible the PEO domains did not contribute much to the thermosensitivity while the PPO domains played the main role in responding to the temperature.

The pH responsive behavior was summarized via normalizing the weight against mass at pH 7 (Figure 60c). The T hydrogel showed normalized weights of 1.22 at pH 2, 1.04 at pH 4, and 0.79 at pH 10. The electrostatic interaction among amino groups in the T hydrogel played a dominant role in influencing the pH responsiveness. At low pH, the repulsion force between the protonated amino groups resulted in the swelling of the hydrogel. On the contrary, at high pH, the lack of electrostatic repulsion force allowed the T hydrogel to shrink. The TED hydrogel exhibited normalized weights of 1.09 at pH 2, 1.03 at pH 4, and 0.65 at pH 10. The density of amino groups in the TED hydrogel is lower than that of the T hydrogel due to the additional JED900. So it is reasonable the swelling of the TED hydrogel at pH 2 is not as significant as the T hydrogel because of weaker repulsion effects among amino groups. At pH 10, the TED hydrogel shrunk at a higher degree than the T hydrogel possibly due to its newly formed crosslinks of intra-

molecular hydrogen bonding considering the great amount of PEO structure [178, 179].

4.3.2.3 Degradation of macro-porous hydrogels

Biodegradability is critical for the tissue regeneration [61]. First, degradation of a scaffold would facilitate the *in vivo* drug release in the local domains. Second, a degradable foreign material could avoid long-term retention of scaffold and the associated chronic inflammation along with adverse response from the immune system. Third, the degradation at an appropriate rate would benefit the regeneration and construction of the new tissue. Therefore it is crucial to investigate the degradation behavior of our material.

Hydrogen peroxide is one of the common *in vivo* stimuli to trigger biodegradation of materials [180, 181]. The oxidation study was performed with 5 vol% hydrogen peroxide at 37 °C for both hydrogels. According to the visual appearance of the T hydrogel in Figure 61a, the scaffold became transparent in the first 24 h of oxidation and then broke down into an apparent soft piece without defined shape in 72 h. It was observed that in 24 h the T hydrogel opaqueness reduced dramatically while the pore size increased while the connection between adjacent spherical particles narrowed and elongated. In the next 24 h, the spherical particles degraded into small and bulging domains while smooth surface of the scaffold was noticed. Visually the size and transparency of the T hydrogel increased. In the third day, the hydrogel exhibited an irregular shape. The 3D network degraded to a 2D frame. The SEM images in Figure 61b indicated that the degradation of the T hydrogel did not complete in 3 days. The oxidation performed in a fashion of bulk degradation but at micro-scale the spherical domains decomposed in a slightly slower rate than the necking domains probably due to its

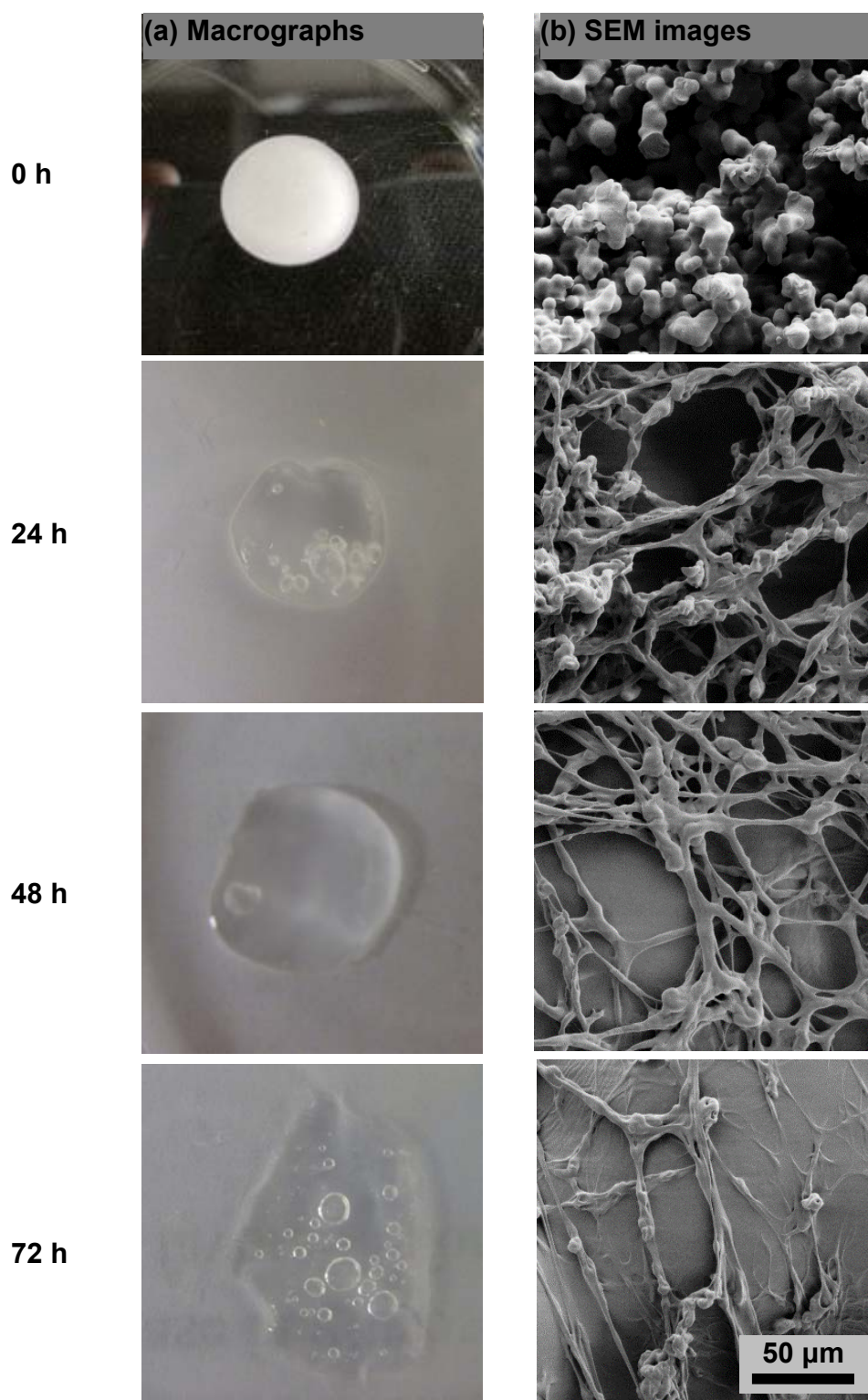


Figure 61 (a) Macrographs and (b) SEM images of the T hydrogel in the presence of 5 vol% hydrogen peroxide at 0 h, 24 h, 48 h, and 72 h.

hydrophobicity, which might influence the penetration of hydrogen peroxide and the diffusion of the oxidized residue.

The visual appearance showed in Figure 62a indicated that the TED hydrogel turned into a completely transparent piece in 4 h in the presence of 5 vol% hydrogen peroxide. The modulus of the TED hydrogel was not monitored using rheometer during oxidation considering the potential corrosion to the equipment. But the rheological properties were estimated by the response of the oxidized piece to the manual shake. The viscosity decreased drastically from the time point of 6 h to 8 h suggesting the total loss of integrity. The SEM images in Figure 62b confirmed that the TED hydrogel no longer kept its structure at macro-scale after first 2 h, and then degraded into free polymers in 8 h.

It is obvious in the comparison of Figure 61 and Figure 62 that the T hydrogel degraded at a much lower rate than the TED hydrogel in response to oxidants. The drastic difference in degradation rate is reasonable considering that the lower crosslink density and higher hydrophilicity of the TED hydrogel allowed the hydrogen peroxide to penetrate more efficient than in the T hydrogel. It suggested the degradation rate could be adjusted via tuning the composition of monomers.

4.3.2.4 Rheology study of macro-porous hydrogels

The modulus of a scaffold is of great significance for its application of neural regeneration [182]. First, the strength of the implant should be comparable with the tissue in local environment [183]. Scaffold with high modulus would introduce pressure on the surrounding tissue and result in stress shielding which would inhibit the neuron migration and neurite outgrowth. On the contrary, low modulus would not necessarily lead to

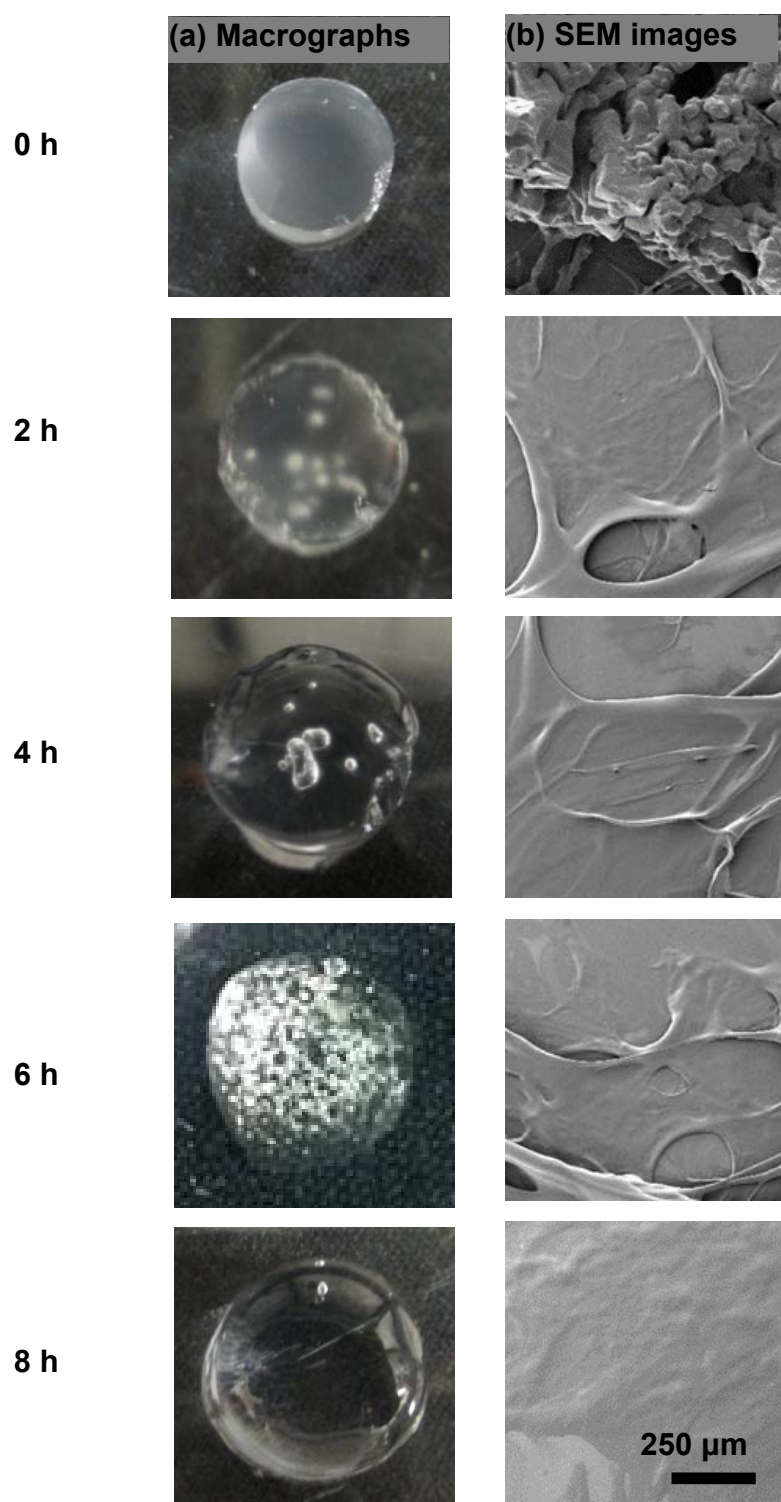


Figure 62 (a) Macrographs and (b) SEM images of the TED hydrogel in the presence of 5 vol% hydrogen peroxide at 0 h, 2 h, 4 h, 6 h, and 8 h.

collapse of the scaffold. In addition, the implant needs to be elastic and potentially expandable to fill the cavity. Second, the stiffness of the scaffold would influence the neuron adhesion and growth [184, 185], and neurite extension [186].

To evaluate the strength, the dynamic modulus was introduced to describe the “stiffness” or viscoelasticity. The dynamic modulus, including storage modulus and loss modulus, can be determined using rheometer via performing reciprocating rotation at different frequencies on pie-shaped samples. The storage modulus represents the elasticity of a sample while the loss modulus indicates its viscosity.

Here our T hydrogel demonstrated a storage modulus around 4 kPa along with a loss modulus ranged from 300 Pa to 1500 Pa (Figure 63a). The hydrogel is in its solid or elastic state because the storage modulus is higher than loss modulus. In addition, the integrity of the T hydrogel is fairly good because the loss modulus shift very little as a function of frequency.

The TED hydrogel showed a storage modulus around 200 Pa and a loss modulus around 30 Pa (Figure 63b). In this figure a transition point at 30 Hz was observed that below 30 Hz the TED hydrogel is dominated by elastic property, and above 30 Hz the gel is dominated by viscous characteristic. It is probably due to the low crosslink density of the TED hydrogel.

The stiffness of the T hydrogel is too high for neural tissues, but the TED hydrogel could be a great candidate for neural regeneration considering its low modulus. For example, the normal human spinal cord tissue shows a storage modulus around 200 Pa. It was also found the neurons showed great activity on the substrate of 50-350 Pa [187]. The storage modulus was studied from 2-10000 Pa in a wider range than that of

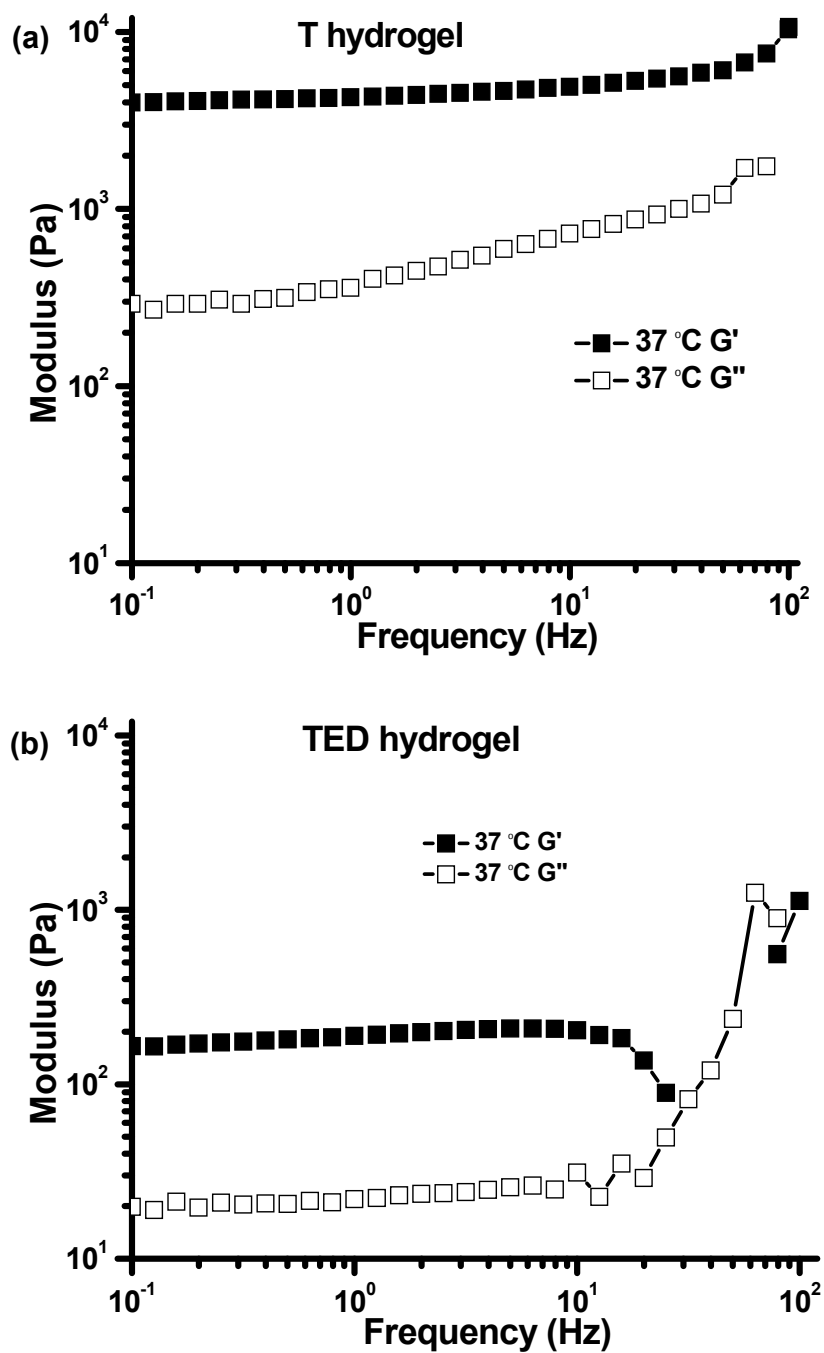


Figure 63 Storage moduli (G') and loss moduli (G'') of the T hydrogel (a) and TED hydrogel (b) as a function of sweeping frequency at 37 °C.

central neuronal system tissue to detect the response of neurons [173, 186, 188, 189]. The softest gel was the favorite of neuronal cells.

4.3.3 Effects of reaction parameters on resultant hydrogels properties

For the T hydrogel, the influence of reaction parameters, such as reactants concentration and reactants ratio, were also investigated. Here the preparation parameters of T prepolymer were kept in consistency, and the reactants mentioned in this section are T prepolymer and PEGDE for the hydrogel fabrication.

The total concentration of T prepolymer and PEGDE was reduced from 15 wt% to 10 wt% while the rest of preparation parameters were maintained. At the concentration of 10 wt%, hydrogel formed with a macro uniform appearance. The micro-structure of 10 wt% T hydrogel was observed using SEM (Figure 64c-d). The 10 wt% T hydrogel exhibited a wider distribution in size of the spherical particles than that of 15 wt% T hydrogel (Figure 64a-b). For example, in Figure 64d, a very small spherical particle was observed with the size around 2 μm attached to a big particle around 10 μm . In addition, the 10 % sample appeared with cavities of great dimension ranged from 30 μm to 50 μm . The lower concentration allowed a lower density of coacervates resulting a less chance for the small coacervates to meet and attach to each other. Therefore the low chance led to a less homogeneous size of the spherical particles.

The dynamic modulus in Figure 65 demonstrated that the storage modulus of 10 wt% T hydrogel was 1000 Pa lower than that of 15 wt%, likely due to the aforementioned increased dimension of cavity.

In addition to the reactant concentration, reactants ratio was paid attention as well. For 15 wt% T hydrogel preparation, the ratio of functional groups between the available

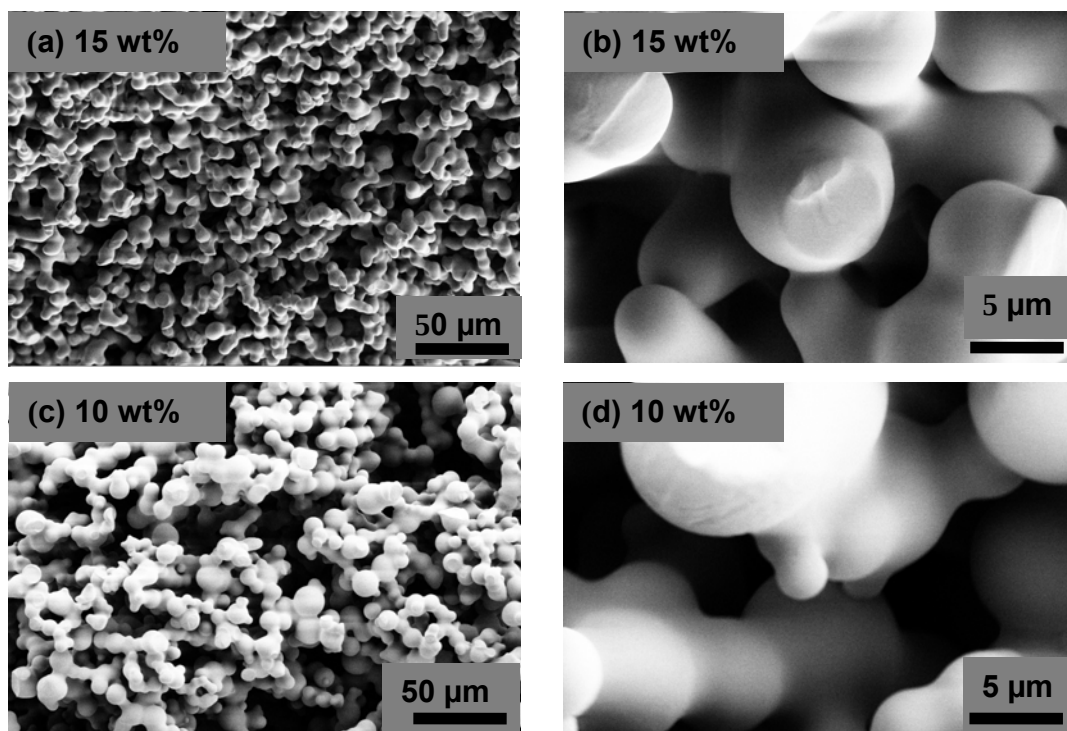


Figure 64 The total concentration of T prepolymer and PEGDE was varied as 15 wt% (a) and (b), and 10 wt% (c) and (d). SEM images of lyophilized T hydrogel were obtained at different magnifications.

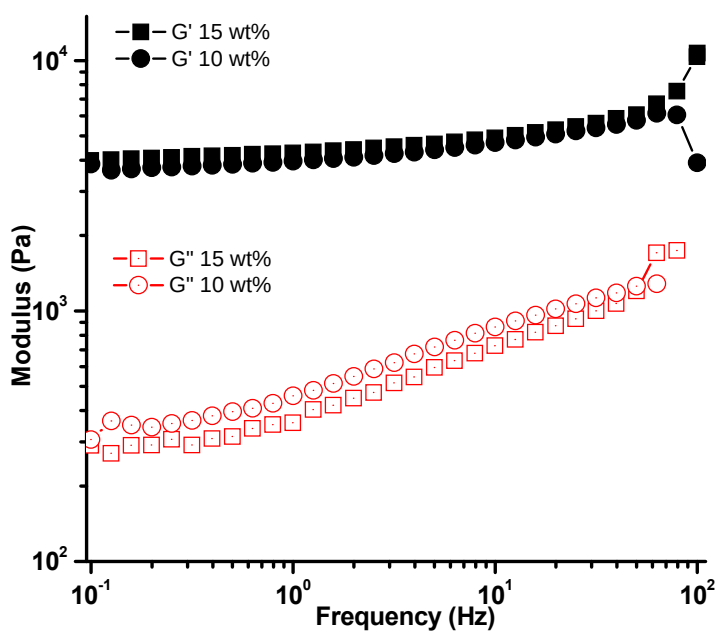


Figure 65 The total concentration of T prepolymer and PEGDE was varied as 15 wt% and 10 wt%. Storage moduli (G') and loss moduli (G'') of the T hydrogels were determined using rheometer.

amino hydrogen of T prepolymer and the epoxide group of PEGDE was 4:3. Here two additional reactants ratios were investigated via increasing and decreasing the molar amount of prepolymer by 25%. Then the functional groups ratios between amino hydrogen and epoxide became 3:3 and 5:3 while the total concentration of reactants was kept consistent at 15 wt%. Hydrogels were obtained at both reactants ratios with apparent homogeneous appearances. After lyophilization the morphologies of the samples were observed using SEM (Figure 66). It was found that in the 3:3 sample large spherical particles around 15 μm were formed along with a very low portion of small particles with diameter of 3 μm . The 5:3 sample was composed of the middle sized spherical particles ranged from 5 μm to 10 μm and small particles with size between 1 μm and 2 μm . Both 3:3 and 5:3 samples demonstrated adequate cavity in the range between 20 μm and 30 μm .

In this system, the number average molecular weight of T prepolymer is around 1,400 Da while the PEGDE is a smaller oligomer of 500 Da. The variance of T prepolymer concentration would change the viscosity of the mixture. For 3:3 sample, the decrease of T prepolymer amount led to reduction of the apparent viscosity of the mixture, and then resulted in a higher mobility of coacervates and a higher chance of aggregation to form large particles. On the contrary, the increase of viscosity caused decrease of coacervates mobility and therefore restricted the growth of spherical particles sizes.

The variance of the reactants ratio did not influence the storage modulus or stiffness of the hydrogels (Figure 67). But the loss modulus of the 3:3 sample was higher than that of 5:3 T hydrogel, because of the enhancement of relaxation behavior of hydrogel due to the additional PEG units in network. In addition, the integrity of both hydrogels was well maintained.

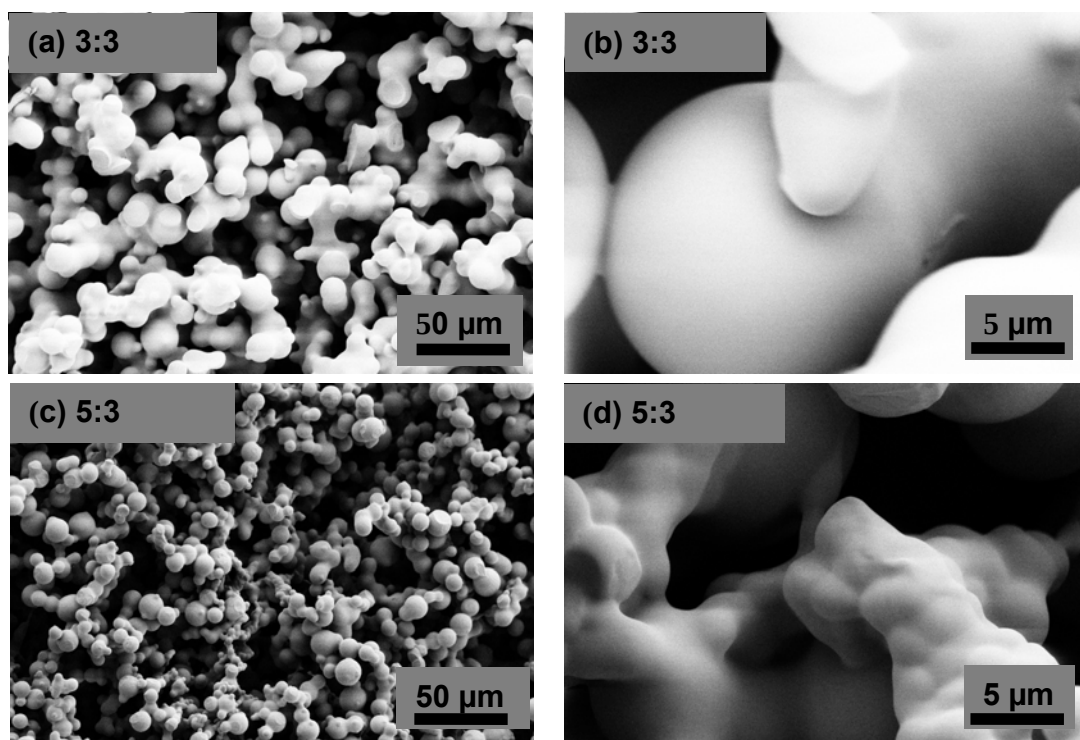


Figure 66 The T hydrogels were prepared with functional groups ratios between amino hydrogen and epoxide at 3:3 and 5:3. SEM images of lyophilized T hydrogels prepared at 3:3 (a) and (b), and 5:3 (c) and (d) at different magnifications.

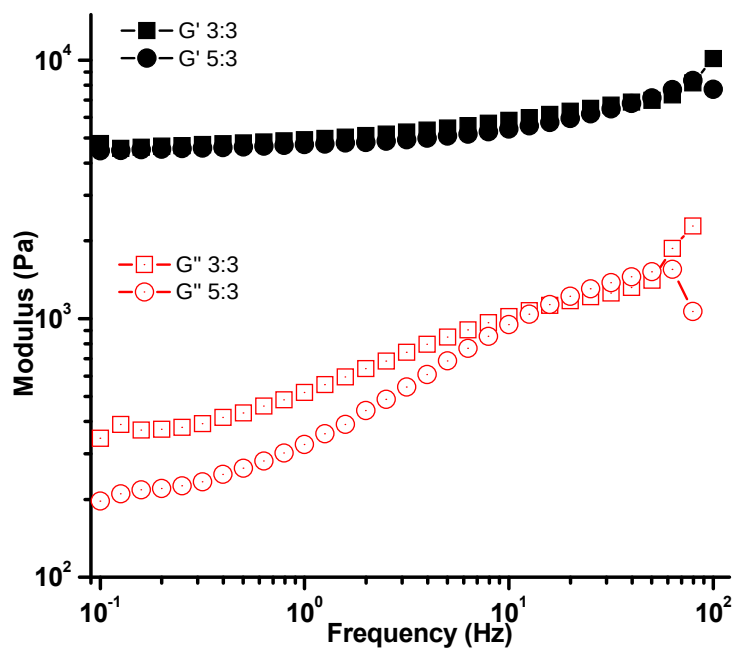


Figure 67 Storage moduli (G') and loss moduli (G'') of the T hydrogel prepared with functional groups ratios between amino hydrogen and epoxide at 3:3 and 5:3.

The cavities and stiffness of hydrogels are critical for the tissue regeneration consider their influences in diffusion of nutrition and metabolites, and attachment, growth, and proliferation of cells. When the reactants concentrations or ratios were varied, the dimension of cavities maintained consistent at the size which facilitates the diffusion of biomolecules. This observation benefits the tuning of hydrogel properties via change of reaction parameters because the influence on the cavity is negligible. For example, stiffness can be reduced via decreasing of reactants concentration while the porous structure retained.

4.3.4 Cytocompatibility of macro-porous hydrogels

The acute cytotoxicity is one of the crucial properties for macro-porous hydrogel as an implant for neuronal regeneration [166]. Here the biocompatibility was investigated via direct contact assay with fibroblast considering its important role in foreign body reaction against implants [32]. In the direct contact assay, a piece of sample will be placed directly onto cells monolayer. During the treatment, leachable chemicals in the sample can diffuse into the culture medium, contact the cell layer, and effect the cell growth. The cytotoxicity of the samples can be evaluated via the morphology and viability of the cells.

The visual observations of the cells after direct contact culture of 24 h with the samples were summarized in Figure 68 as well as the quantitative data obtained from WST-1 assay (Figure 68f). In Figure 68a-c, the shady areas above the dash lines were the (a) TED hydrogel, (b) T hydrogel, and (c) negative control PTFE sheet, respectively. It was observed that the cell densities in Figure 68a-c were close to confluency and comparable to the control (Figure 68d) [190]. Furthermore, the morphologies of the

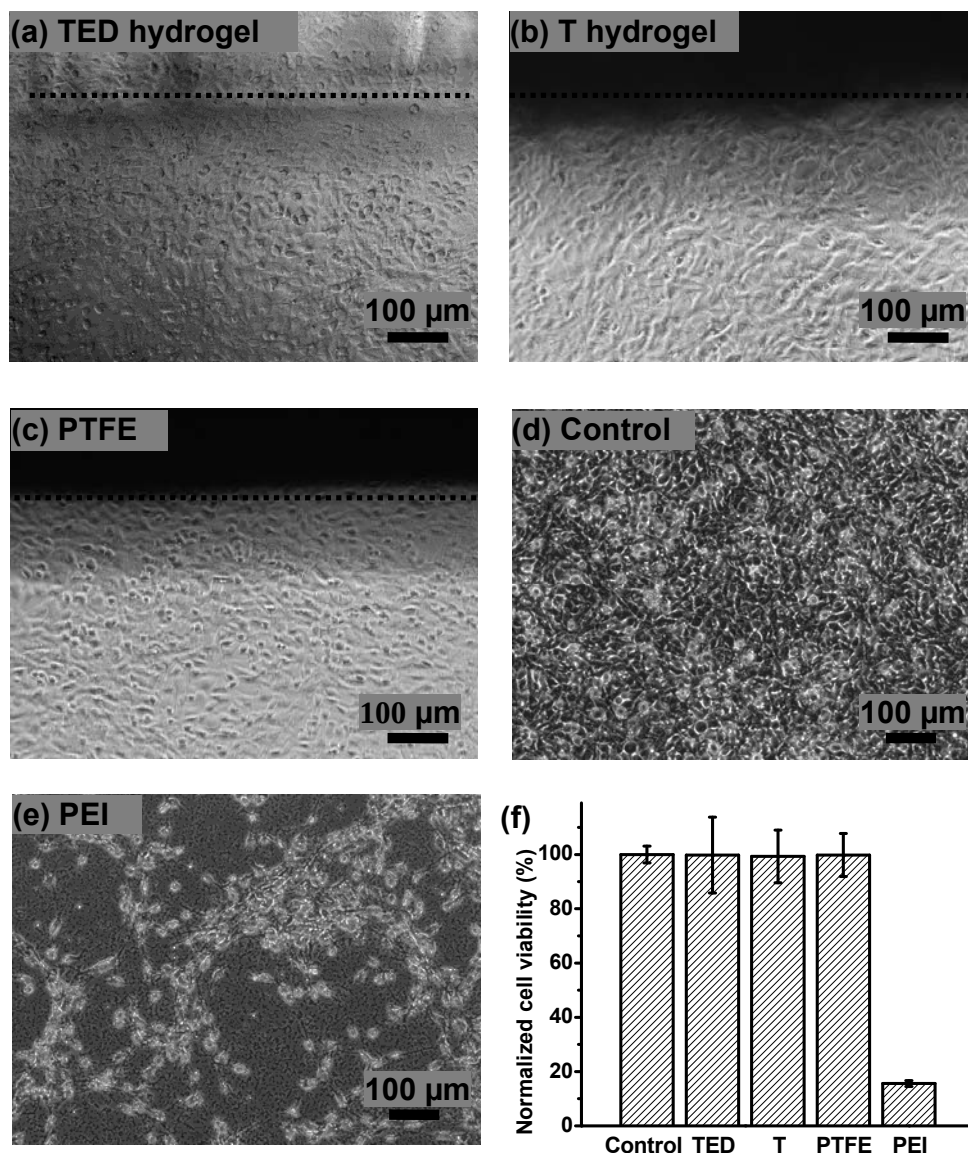


Figure 68 Biocompatibility of hydrogels via direct contact study. Optical images (a-e) and normalized viability (f) of cells after 24 hours of contact with the T & TED hydrogels, PTFE plate (negative control), and PEI (50 $\mu\text{g/mL}$, positive control) as well as untreated control. The dash line in (a-c) showed the boundaries of samples. In the images of (a-c), the cells above the lines were covered by the samples.

fibroblasts in Figure 68a-d appeared in a regular shape without rounded cells. The cell morphologies and densities suggested there was no obvious malformation, degeneration or lysis during the treatment [191]. In addition, the positive control was examined with 50 $\mu\text{g/mL}$ PEI indicating a low cell density along with cell debris. The visual observations were confirmed by the quantitative data presented in the bar graph (Figure 68f) corresponding to the high cell survival in direct contact with TED and T hydrogels. Thus, both T and TED hydrogels exhibited good cytocompatibility demonstrated by direct contact assay.

4.3.5 In vitro neuronal response to macro-porous hydrogels

The biological activity of hydrogel is of great significance in providing favorable chemical and physical microenvironment for neurons attachment, survival, proliferation, differentiation and neurite extension [192]. Here cortical neurons were used to examine the bioactivity of the T & TED hydrogels. The morphology of neurons was observed using fluorescent microscope via labeling the live cells with a green stain (calcein AM) and SEM via fixation and lyophilization. The fluorescent images of live stained neurons were collected, inverted and processed into binary images for a straightforward presentation of distribution and morphology of cells. For the T hydrogel, the image shown in Figure 69a indicated 4 h after immobilization, small, rounded neuronal cells were observed scattered throughout the scaffold suggesting that attachment and survival of neurons. In 24 h the Figure 69b demonstrated that clumps of cells formed while the numbers of individual neurons decreased dramatically. The size of the clumps increased in the next 24 h along with the number of individual cells. The neurites, which were not obvious in the binary images, were observed in the SEM samples (Figure 70). It was

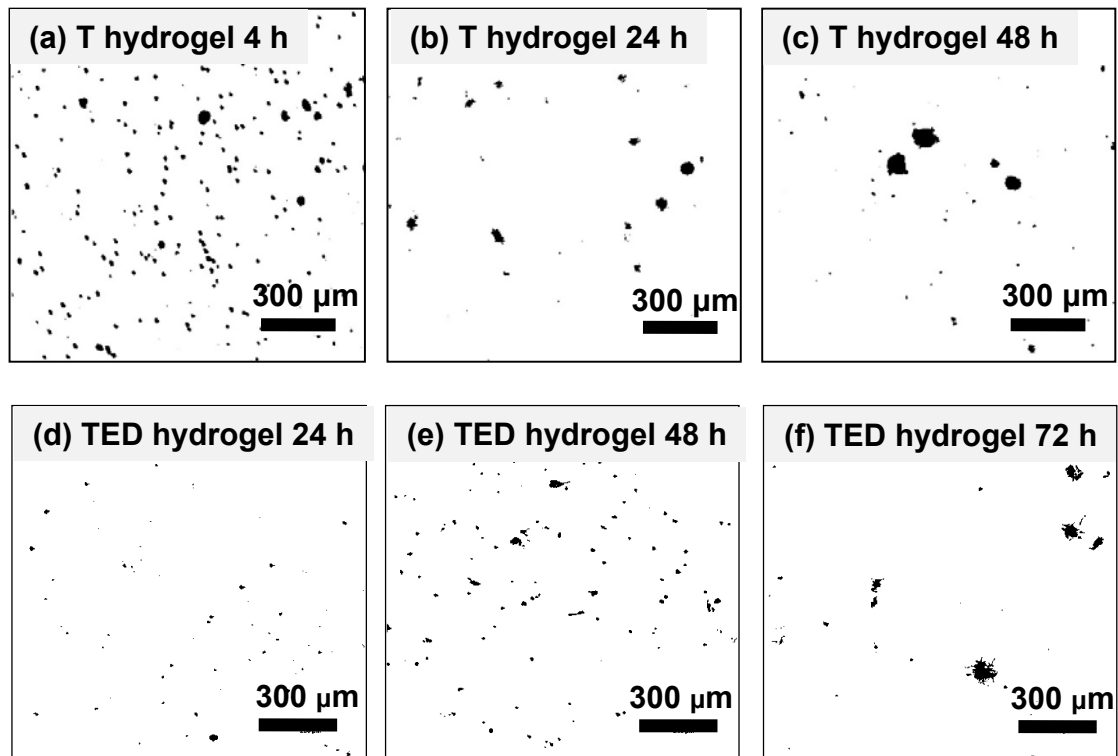


Figure 69 The fluorescent signals of live cells were collected, inverted and processed to generate binary images of cortical neurons after 4 h (a), 24 h (b), and 48 h (c) incubation on the T hydrogel, after 24 h (d), 48 h (e), and 72 h (f) incubation on the TED hydrogel.

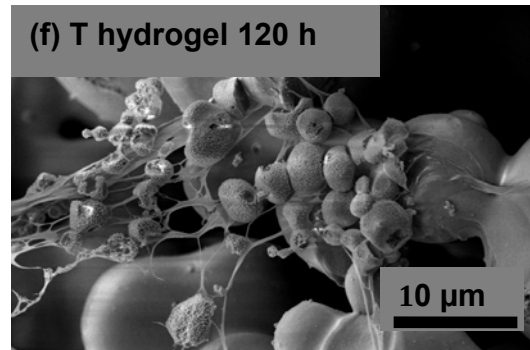
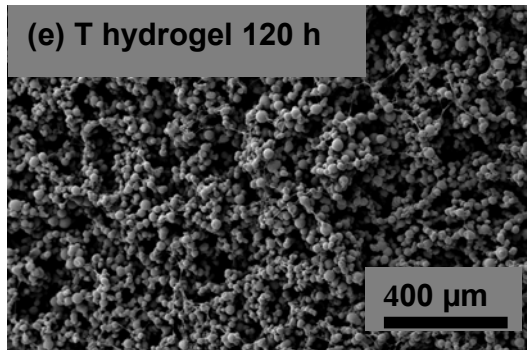
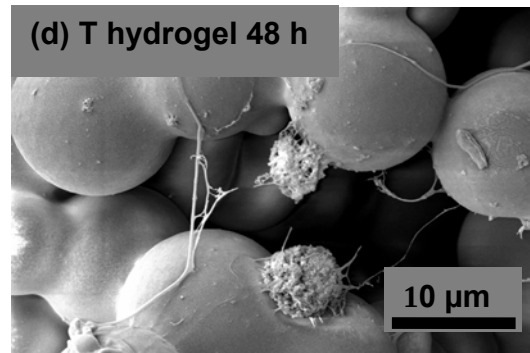
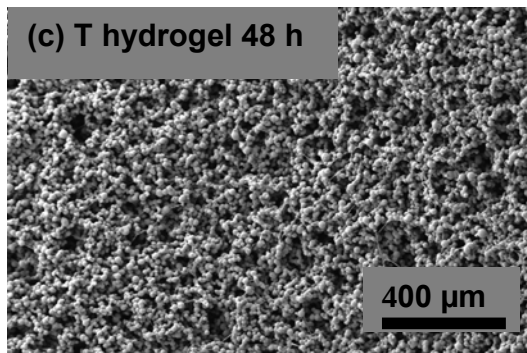
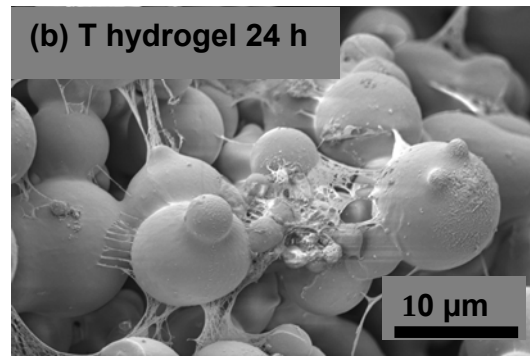
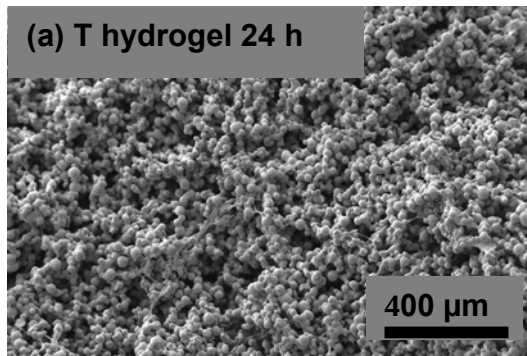


Figure 70 SEM images of cortical neurons after 24 h (a) and (b), 48 h (c) and (d), and 120 h (e) and (f) incubation on the T hydrogel.

noticed that there was clear neurite outgrowth in 24 h (Figure 70a-b), increased amount of neurites in 48 h (Figure 70c-d), and significant amount of neurites in 120 h (Figure 70e-f). The number and length of the neurites extensions increased as a function of time.

For the TED hydrogel, it was observed that the neurons attached to the hydrogel at very low density in the first 24 h (Figure 69d). In the next 24 h, the numbers of attached live cells increased significantly while clumps of small size were noticed as well (Figure 69e). In 72 h, the clumps of neurons increased in size significantly along with the drastic decrease of number of individual cells (Figure 69f). Outgrowth of short neurite extensions were noticeable from the clumps in 72 h. The SEM image in 24 h (Figure 71) proved that the outgrowth of neurite extensions from individual cells. The neurite seemed being embedded into the gel because of drastic collapse of the TED hydrogel during dehydration.

Both hydrogels are favored by the neurons in attachment, survival, and neurite extension. The hydrophobic domains such as spherical particles in T hydrogel and granules in TED hydrogel provided substrate for neurons to attach and adhere. The amino groups which were positively charged at physiological pH benefited the neuron survival and neurite outgrowth. The time for attachment to the TED hydrogel appeared to be about 48 h longer than that to the T hydrogel. This delay of attachment is probably caused by the lower positive charge density of the TED hydrogel due to the addition of JED900.

4.4 Conclusion

Macro-porous hydrogels were successfully fabricated via thermally-induced phase separation of prepolymers toward the application for neuronal regeneration. The T

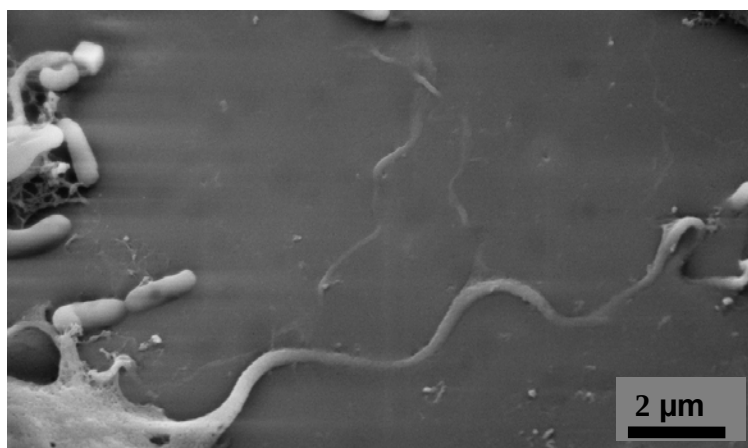


Figure 71 SEM image of cortical neural cells after 24 h incubation on the TED hydrogel.

hydrogel showed a uniform structure with conjoint spherical particles while the TED hydrogel exhibited a homogeneous network of thick branches containing scattered small spherical granules. Both hydrogels provide micro-sized interconnected cavity allowing diffusion of nutrition and metabolites, and migration of cells. The dynamic modulus of TED hydrogel was determined to be compliant to neuron growth and differentiation. In addition, hydrophobic domains with tunable density in the hydrogels are promising for delivery of hydrophobic drugs, while the cationic amino groups benefit the neurite outgrowth. Biodegradation could be performed at a proper rate in the presence of a common oxidant hydrogen peroxide. Above all, the hydrogels exhibit great biocompatibility in direct contact assay with fibroblast, and favorable microenvironment for cortical neuronal cells, indicating a probable use for neuronal regeneration.

Chapter 5 Summary and Future Work

Polymeric materials capable of responding to multiple stimuli were designed in different forms including micro/nano-sized particles, nano-scale multilayers, and macro-porous 3D scaffolds. Chemical compositions and structures were characterized for understanding reaction mechanism and optimizing preparation parameters. Physical properties, such as dimensional size and morphology, surface charge density, hydrophobicity, molecular weight, and lower critical solution temperature were qualitatively or quantitatively determined. Responsiveness of materials were investigated in detail toward variance of temperature and pH, and the presence of oxidative stress. For biomedical application, the cytocompatibility and bioactivity of the obtained materials were studied using cell line and primary cells respectively. The loading capacity of the vehicles toward hydrophobic molecules were examined using fluorescent dyes as model molecules.

The specific conclusions in this dissertation were listed below:

1. A series of polyetheramine based micro/nano-sized gel particles were successfully fabricated using commercially available Jeffamines and 1, 3-butadiene diepoxide via thermally induced phase separation and ring-opening reactions.
2. The physical properties of the synthetic particles, including particle size, surface charge density, and swelling ratios are tunable via preparation parameters such as monomer ratio, monomer concentration, reaction temperature and reaction time.
3. The obtained micro/nano-sized gel particles exhibited multi-responsiveness toward temperature, pH, and oxidative stress, which could be applied for drug release.

4. The cytotoxicity of gel particles is dose and surface charge density dependent. Reduction of surface charge density can be achieved via tuning the monomer composition for improvement of biocompatibility.

5. The drug loading capability of obtained particles was proved using Nile red, and triggered release was demonstrated with the application of hydrogen peroxide.

6. A novel vinyl monomer 3-(*tert*-butoxycarbonylmethyl)-*N*-vinyl-2-caprolactam was successfully synthesized using *N*-vinyl-2-caprolactam and *tert*-butyl bromoacetate via nucleophilic substitution facilitated by a lithium enolate intermediate.

7. The copolymers MCOOH-PVCLs were successfully prepared via copolymerization of 3-(*tert*-butoxycarbonylmethyl)-*N*-vinyl-2-caprolactam and *N*-vinyl-2-caprolactam followed by deprotection of the *tert*-butoxycarbonyl group to expose carboxylic acid group.

8. The copolymers MCOOH-PVCLs demonstrated pH-dependent thermoresponsive behaviours with higher pH dependence at corresponding monomer compositions than COOH-PVCLs which was fabricated from 3-(*tert*-butoxycarbonyl)-*N*-vinyl-2-caprolactam and *N*-vinyl-2-caprolactam.

9. The capability of layer-by-layer assembling of the copolymer was proved using polyethylenimine as the positively charged polyelectrolyte via electrostatic interaction.

10. Hydrogels with homogeneous micro-sized interconnected cavity were successfully prepared via thermally induced phase separation of polyetheramine containing prepolymer. The T hydrogel showed a uniform structure with conjoint

spherical particles while the TED hydrogel exhibited a homogeneous network of thick branches containing scattered small spherical granules.

11. The hydrogels exhibit good biocompatibility in direct contact assay with fibroblast, and drug loading capability using Nile red as the model molecule.

12. The hydrogels could provide favorable microenvironment for neurons considering the neuronal response including attachment, survival, and neurite outgrowth.

Based on the above conclusions in this dissertation, future work is proposed, including:

1. Delivery of hydrophobic therapeutic biomolecules and gene fragments such as DNA or RNA using micro/nano-sized gel particles.

2. Tuning the chemical structure of 3-(*tert*-butoxycarbonylmethyl)-*N*-vinyl-2-caprolactam via addition of one more methylene group to investigate the influence on phase transition profile of its copolymers. The potential reagent to replace *tert*-butyl bromoacetate could be *tert*-Butyl 3-bromopropionate and *tert*-Butyl α -bromoisobutyrate.

3. Loading biomolecules such as growth factor in macro-porous hydrogels and examine the effects on neural culture.

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Vita

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