Intelligent Nano/Microgels for Cell Scaffold and Drug Delivery System

Jing Shen
Graduate Center, City University of New York

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Intelligent Nano/Microgels for Cell Scaffold and Drug Delivery System

By

Jing Shen

A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Yujia Xu

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK
Abstract

Intelligent Nano/Microgels for Cell Scaffold and Drug Delivery System

By

Jing Shen

Adviser: Professor Shuiqin Zhou

Stimulus-responsive polymer microgels swell and shrink reversibly upon exposure to various environmental stimuli such as change in pH, temperature, ionic strength or magnetic fields. Therefore, they become ideal candidates for biomaterial applications. For this work, we focus on the several intelligent microgels and their application on two areas: the cell scaffold and the drug delivery system.

As for the cell scaffold, it can be realized by colloidal supra-structure microgels, which constructed by the thermo-driven gelation of the colloidal dispersion of poly(N-isopropylacrylamide-co-acrylamide) poly(NIPAM-co-AAm) microgels (Chapter 3). Such microgels exhibit a reversible and continuous volume transition in water with volume phase transition temperature (VPTT) \(\approx 35^\circ C\) and remain partially swollen and soft under physiological conditions. More importantly, the size of the microgel particles can affect the sol-to-gel phase transition of the microgel dispersions, alter the syneresis degree of the constructed colloidal supra-structures, and tailor the cytocompatibility. The constructed colloidal supra-structure can be regarded as a model system for a new class of cell scaffolds.

As for drug delivery system, Chapter 4 and Chapter 5 focus on the development of biocompatible microgels–based systems for delivering a traditional anti cancer drug–curcumin.
These thermo-responsive core-shell structure microgels are constructed from oligo(ethylene glycol) as a hydrophilic shell and hydrophobic biocompatible materials as core, such as poly(2-vinylanisole) and poly(4-allylanisole). The rationally designed core chain networks can effectively store the hydrophobic curcumin drug molecules via hydrophobic interactions, thus provide high drug loading capacity; while thermo-sensitive nonlinear poly(ethylene glycol) (PEG) gel shell can trigger the drug release by local temperature change, offering sustained drug release profiles. In Chapter 5, additionally embedded of magnetic Fe₃O₄ nanoparticles enable such hybrid nanogels to delivery pharmaceuticals to a specific site of the body by applying a gradient magnetic field.

Chapter 6 investigated a class of well-defined glucose-sensitive microgels as an insulin drug release carrier, obtained via polymerization of 4-vinylphenylboronic acid (VPBA), 2-(dimethylamino) ethyl acrylate (DMAEA), and andoligo(ethylene glycol)methyl ether methacrylate (MEO₅MA). The presence of MEO₅MA monomer could retard the glucose-sensitive network from swelling because the rapid hydrogen bonding between the glucose molecules and the ether oxygens of the MEO₅MA is prior to the glucose binding to the PBA groups. Therefore, the set point of glucose sensitivity of microgels could be adjusted possibly and result in potential biomedical applications. Compared to the non-imprinted copolymer microgels, the glucose imprinting of the microgels can create and rigidly retain more binding sites complementary to the shape of the target glucose molecule in the crosslinked polymer network, thus improve the sensitivity and selectivity of the microgels in response to the glucose level change. Additionally, the introduction of fluorescent Ag nanoparticles (NPs) to the microgels can realize the integration of optical glucose detection and self-regulated insulin delivery into a single nano-object.
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<td>AAm</td>
<td>Acrylamide</td>
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<tr>
<td>AAPH</td>
<td>2,2′-azobis(2-methylpropionamidine) dihydrochloride</td>
</tr>
<tr>
<td>AO</td>
<td>Acridine Orange</td>
</tr>
<tr>
<td>EB</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
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<tr>
<td>BIS</td>
<td>N,N'-methylenebisacrylamide</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>CSM</td>
<td>Core-shell Microgels</td>
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<tr>
<td>DLS</td>
<td>Dynamic Laser Scattering</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
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<tr>
<td>DVB</td>
<td>Divinylbenzene</td>
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<tr>
<td>EDC</td>
<td>N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide Hydrochloride</td>
</tr>
<tr>
<td>EGDM</td>
<td>Ethylene Glycol Dimethacrylate</td>
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<tr>
<td>MEO(_3)MA</td>
<td>Oligo(ethylene glycol)methyl ether methacrylate ((M_n = 300) g/mol)</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>PANI</td>
<td>Polyaniline</td>
</tr>
<tr>
<td>PBA</td>
<td>Phenylboronic Acid</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEGDMA</td>
<td>Poly(ethylene glycol) Dimethacrylate</td>
</tr>
<tr>
<td>PL</td>
<td>Photoluminescence</td>
</tr>
<tr>
<td>PMAA</td>
<td>Polymethylmethacrylate</td>
</tr>
<tr>
<td>PNIPAM</td>
<td>Poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>PRINT</td>
<td>Particle Replication Inn Non-wetting Templates</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>PSD</td>
<td>Particle Size Distribution</td>
</tr>
<tr>
<td>PVAS</td>
<td>Poly(2-vinylanisole)</td>
</tr>
<tr>
<td>QDs</td>
<td>Quantum Dots</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SFEP</td>
<td>Surfactant-Free Emulsion Polymerization</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>VPBA</td>
<td>4-Vinylphenylboronic acid</td>
</tr>
<tr>
<td>VPTTT</td>
<td>Volume Phase Transition Temperature</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction of Responsive Polymer Microgels

1.1 Introduction of Microgels

Aqueous microgels are an important part of water-borne polymer colloids and have been widely employed in coating industry, agriculture, food processing, and medicine formulation to modify rheological properties, to retain water, and for many other purposes\(^1\). There is no universal definition of microgels; however, some special properties can be described. Aqueous microgels are mostly spherical particles with average diameter between 50 nm and 5 \(\mu\)m. They are stabilized in continuous medium and form stable dispersions. Microgels are characterized by a degree of swelling, an average cross-linking density, and characteristic time constants for swelling and shrinking\(^1\). The swelling degree depends upon the interplay between polymer-polymer and polymer-water interactions and the crosslinking degree. Swelling occurs when ionic repulsion and osmotic forces exceed attractive forces, such as hydrogen bonding, van der Waals interactions, hydrophobic and specific interactions among the polymer chains\(^2, \ 3\). Numerous factors can influence the behavior of polymer chains inside the microgel, such as temperature, pH\(^4\), ionic strength, electric field\(^5\), and etc.. By taking into account these features, aqueous microgels can be considered as porous crosslinked polymeric particles that swell in water and
adjust their dimensions, densities, and related properties according to the surrounding conditions\textsuperscript{6}. (Figure 1–1)

![Figure 1–1](image)

**Figure 1–1** Schematic of change in microgel size in response to environmental stimuli\textsuperscript{2}.

In the last decade, numerous stimuli-sensitive polymers have been applied in the design of microgel systems. One of the widely studied stimuli-sensitive polymers is poly(N-isopropylacrylamide) (PNIPAM)\textsuperscript{7}. The PNIPAM microgel has been first reported by Philip Chibante, a high school summer student, under Dr. Pelton’s supervision in 1978\textsuperscript{8}. This microgel exhibits endothermic entropy-driven phase transition in water\textsuperscript{9}. The amide side chains of PNIPAM form hydrogen bonds to water, while the isopropyl group induces hydrophobic structure and this leads to entropy-controlled polymer-polymer interactions. The PNIPAM chains exhibit a random-coil structure if the solvent-polymer interactions are stronger than the polymer-polymer interactions. If the hydrogen bonds between water and the PNIPAM chains break because of the increased temperature, polymer-polymer interactions become dominant, leading to a coil-globule transition. The temperature at which this phase separation occurs is called the lower critical solution temperature (LCST) of a polymer\textsuperscript{10}. The microgels of crosslinked PNIPAM chains exhibit a thermoresponsive volume phase transition temperature (VPTT) close to the LCST of non-crosslinked PNIPAM chains\textsuperscript{11}. PNIPAM-based microgels will shrink if the temperature is above its VPTT and swell at the temperature below its VPTT.
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Compared to bulk gels, microgels have several advantages. First, they can respond very quickly to the environmental change. Second, the mono-dispersed microgel particles, as building blocks, can be readily assembled to meet various application requirements. Third, they can provide potential biocompatibility and their surface can be conjugated to receptor-specific molecules to achieve targeting ability. Therefore, microgels have attracted much attention for applications in materials and biomedical fields during the past several decades.

1.2 Microgel Synthesis

1.2.1 Synthetic Routes

Aqueous microgels can be prepared by three synthetic strategies: (1) physical self-assembly or chemical crosslinking of prepolymerms in homogeneous phase or in microdroplets; (2) polymerization of monomers in homogeneous phase or in microdroplets; (3) photolithographic techniques.

Physical or chemical crosslinking of polymers can be realized in water or in a water-in-oil (W/O) emulsion system. The former strategy is usually used to prepare chitosan-based microgels. Reversible physical crosslinking of chitosan and polyethyleneimine or tripolyphosphate based on electrostatic interactions leads to formation of pH-sensitive microgels. In later case, aqueous droplets of prepolymerms are stabilized by oil-soluble surfactants in a continuous oil phase. For example, hyaluronan-based microgels were synthesized by crosslinking of carboxylic units of hyaluronan with adipic dihydrazide in aqueous droplets.

The polymerization (free radical or controlled radical) of monomers in the presence of a crosslinking agent in aqueous phase is most popular these days, which can be divided into two techniques: precipitation polymerization and emulsion polymerization.
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For precipitation polymerization, all ingredients are dissolved in a solvent (water) to form a homogeneous mixture, then initiation of polymerization takes place. The formed polymers are transformed into a collapsed state because the reaction temperature is far above its VPTT and become crosslinked by crosslinker agent, forming colloidal polymer network microgels. We will describe the detail of this technique later.

The term emulsion polymerization encompasses several related processes: (1) conventional emulsion polymerization, (2) inverse emulsion polymerization, (3) miniemulsion polymerization, (4) dispersion polymerization, and (5) microemulsion polymerization. Conventional emulsion polymerization accounts for the majority of the world’s production of commercial polymers, such as polystyrene (PS). The conventional emulsion polymerization is carried out in an oil-in-water emulsion (Figure 1–2), in which surfactant is dissolved in water until micelle has been formed, droplets of monomer (the oil) are emulsified by surfactant in a continuous phase of water. The polymerization will take place in the micelle when free radicals migrate into the micelle and react with monomers. Monomer migrates in the large monomer droplets of the micellar core to sustain polymerization until all the monomer is consumed.

![Figure 1–2 Mechanism of emulsion polymerization](image)

Figure 1–2 Mechanism of emulsion polymerization.
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The photolithographic technique has been recently used on solid substrates for preparation of hydrogel layers with variable thickness\textsuperscript{19}. The PRINT (particle replication in non-wetting templates) technique utilizes elastomeric molds from a low surface energy perfluoropolyether network\textsuperscript{20, 21}. The molds prevent the formation of an interconnecting film between molded objects and allow production of monodisperse microgel particles of different sizes, shapes, compositions, and surface functionalities.

1.2.2 Precipitation Polymerization

Precipitation polymerization is probably the most frequently used technique for microgel synthesis. We used this technique to synthesize the micro-/nanogels used in our projects. The mechanism of free radical precipitation polymerization has been demonstrated in Figure 1–3. In such system, all ingredients, including the monomer(s), crosslinker, and initiator, are dissolved in water. A homogeneous nucleation mechanism leads the formation of microgel particles. One initiator molecule (peroxide- or azo-based compound) decomposes into two free radicals under reaction temperature (50–70 °C), which can initiate polymerization in the homogeneous solution phase; therefore, the water-soluble oligomers start to grow. As oligomers reach a critical chain length, the growing chain condenses to form precursor particles because the phase separation occurs at the polymerization temperature higher than the LCST of the polymer. The precursors can grow by different mechanisms. Precursor particles can (1) aggregate with other precursor particles to form a larger colloidally stable polymer particles; (2) deposit onto an existing, colloidally stable microgel particle; or (3) grow by addition of monomers or macroradicals. Surfactant is used to prevent precursors from aggregating; thus the precursors formed in the early stage of reaction can be stabilized to form nuclei particles. The more surfactant, the more nuclei
particles will be formed. The growing chains prefer to deposit onto these existing precursor particles. Hence, the higher the concentration of surfactant, the smaller the resulting gel particle size is. Besides surfactants, the growing polymer particles can also achieve colloidal stability by the presence of electrostatic stabilization provided by the ionic groups originating from the initiator. At this time, microgel particles are in a collapsed state but still contain a lot of water. When the polymerization is completed and the reaction mixture cools down to room temperature (below LCST of polymer chains), microgel particles swell and develop a “hairy” morphology. Microgels are stabilized by steric mechanisms because of the formation of hydrogen bonds between polymer segments and water molecules.

![Mechanism of precipitation polymerizations.](image)

**Figure 1–3** Mechanism of precipitation polymerizations.

The precipitation polymerization is a versatile technique and offers several advantages for the preparation of aqueous microgels.

1. The polymerization process can be carried out as a batch, semibatch, or continuous process.
2. Microgel size can be controlled over a broad range (from tens of nm to 3 μm) by the use of surfactants or co-monomers.
3. Microgel particles with narrow particle size distribution (PSD) can be prepared.
4. Copolymer-based microgel can be prepared by integrating different co-monomers into the microgel network during the polymerization process.

5. Hybrid colloids can be obtained by encapsulation of nanoparticles (NPs) during microgel formation.

However, precipitation polymerization has some limitations:

1. Because of the high polymerization temperature, only thermally stable materials can be used, which limits the incorporation of delicate biomacromolecules.

2. It is difficult to prepare very small microgels (size below 50 nm) without use of additional stabilizing agents.

3. Formation of soluble fraction during the polymerization process.

1.3 Microgel Properties

1.3.1 Microgel Structure

The internal structure of microgels particles influences its swelling properties. The most important structural information is the distribution of cross-linked monomers as a function of distance from the particle center. The crosslinking density usually decreases from the center of the particles toward the periphery. Nieuwenhuis et al. examined polymethylmethacrylate (PMMA) microgel particles cross-linked with divinylbenzene (DVB) and ethylene glycol dimethacrylate (EGDM) dispersed in benzene. The results from laser light scattering and viscometry techniques showed that the microgel particles had different diameters and possessed a heterogeneous distribution of cross-links. Mcphee et al. proposed that a large proportion of the cross-links were incorporated during the initial growth of particles by measuring the efficiency of cross-linking monomer incorporation into growing particles during surfactant-free
emulsion polymerization (SFEP)\textsuperscript{24}. This is because the solubility of a polymer chain decreases with increasing molecular weight and addition of cross-linking monomer facilitates a substantial increase in its length. Accordingly, attainment of uniformly swollen microgel particles may be more likely using polymerization in a good solvent.

1.3.2 Microgel Swelling Theory

The swelling of microgel particles in organic solvents has been described by using Flory’s theory of network swelling\textsuperscript{25}. A polymer network immersed in a good solvent imbibes solvent in order to balance the chemical potential inside and outside the gel network. At the same time, the presence of cross-links restricts the extent of swelling. Therefore, swelling continues until the sum of the elastic forces between cross-links in equal to osmotic force. The extent of network swelling is usually described by the polymer volume fraction ($\phi_2$) obtained at equilibrium ($\phi_2 = 1$ in the collapsed state). Flory’s theory leads to\textsuperscript{25}:

$$\phi_2 = \left( \frac{X_{12}}{V_c \chi_{12} - x_{12}} \right)^{3/5}$$

where $X$ is the number of cross-links present within a collapsed network volume $V_c$. The subscripts 1 and 2 refer to the solvent and network polymer, respectively; $\nu_1$ is the molar volume of the solvent and $x_{12}$ is the Flory solvent-polymer interaction parameter. The term ($x/V_c$) represents the average density of cross linked units in the collapsed particle.

There is usually a high proportion of non-cross-linking polymer segments (monomer B) contained in microgel particles as well as a minor proportion of (normally) difunctional cross-linking segments (A). The mole fraction of the latter ($X_A$) is typically less than 0.1. If it is assumed that the molecular weights of A and B segments are the same and each mole of di-
functional cross-linking monomer introduces two moles of cross-linked units, it is readily shown that:

\[
\frac{X}{V_c} = \frac{2x_A \rho_B}{M_B} \quad (1-2)
\]

where \(M_B\) and \(\rho_B\) are the molecular weight and density of the B segments, respectively.

Substitution of Eq. (1–2) into Eq. (1–1) and assuming \(\rho_B = 1\) leads to a simple expression describing the dependence of the volume fraction of microgel particles on the network composition and solvency:

\[
\phi_2 = \left\{\frac{2x_A V_1}{v_{12} M_B}\right\}^{3/5} \quad (1-3)
\]

where the excluded volume parameter \(v_{12} = (0.5 - x_{12})\). The polymer volume fraction increases (particles de-swell) if the cross-link density increase of the solvency becomes poorer (\(v_{12}\) decreases).

Flory’s theory of network swelling has been applied to polystyrene microgel particles dispersed in ethylbenzene and was extended to consider the effect of added non-adsorbing (free) polymer. The effective concentration of incorporated cross-linking monomer has been found to be less than that actually used during the preparation, thus a significant proportion may not have reacted.

There are two drawbacks to Flory’s theory of gel swelling. First, it assumes a uniform cross-link density within the network. The cross-linked density is more likely decreased from the center of the particles toward the periphery (as discussed in Section 1.3.1). Second, the concentration dependence of \(\chi\) is not included. There might be a problem with aqueous gel systems related to hydrogen bond and hydrophobic bonding, because the segment/water concentration varies with swelling.
1.3.3 Stimuli-Responsive Behavior

1.3.3.1 Temperature

Figure 1–4 a) Illustration of typical thermoresponsive behavior of PNIPAM microgel particles; b) associated change in hydrodynamic diameter of PNIPAM microgel particles as a function of temperature$^{30}$. 

In Figure 1–4a, the typical thermoresponsive behavior of PNIPAM has been illustrated. At room temperature (~25 °C), the particle is swollen due to extension of the polymer networks toward a soft porous structure$^{31}$. When microgels are immersed in a good solvent, solvent molecules can penetrate into the polymer structure until the chemical potential balance between inside and outside. Thus, microgel particles swell until the elastic forces of the cross-links that restrict swelling are equal to the osmotic force imposed by the solvent$^{32}$. When the temperature
increases, the particle collapses, becoming a tightly packed structure more like a hard particle such as a polystyrene latex\textsuperscript{33}.

Figure 1–4b shows a typical plot of the change in microgel particle size (hydrodynamic diameter) as a function of temperature. The temperature at which the particle switches between swollen and collapsed conformations is volume phase transition temperature (VPTT), which is usually considered being the midpoint of the transition curve using particle diameter versus temperature\textsuperscript{34}.

1.3.3.2 pH

Figure 1–5 Conformational changes of a polyelectrolyte microgel as a function of pH\textsuperscript{32}.

Microgel particles containing acidic or basic functional groups (e.g. carboxylic acid, amines etc.) exhibit a pH-dependent swelling/shrinking behavior. Figure 1–5 shows the conformational change of pH sensitive microgel driven by change of pH. The mechanism of swelling of pH-responsive particles is explained by the internal osmotic pressure due to the mobile counter-ions contained within the particles, which balance the internal electrostatic repulsion\textsuperscript{35}. When the pH rises above the pK$_a$ of the acid groups within the microgel structure,
the polymer network chains become ionized and repel each other, resulting in the swelling of the particles. At the pH below the pK_a, the deionized groups facilitate the adoption of a more compact structure of the polymer chains\textsuperscript{32, 36}. Additionally, by combining a pH-sensitive co-monomer such as acrylic acid (AA) with a temperature-sensitive monomer such as NIPAM, it is possible to prepare microgel particles sensitive to both pH and temperature\textsuperscript{36}.

### 1.3.3.3 Ionic

![Figure 1-6](image)

**Figure 1-6** Hydrodynamic diameter of poly(NIPAM-co-AA) (95:5\%) microgel particles as a function of ionic strength of NaCl at pH 6 and 25 °C at a microgel concentration of 0.07\% (w/w)\textsuperscript{36}.

The size and stability of microgel particles prepared from the copolymerization with ionizable monomers can be influenced by the presence of electrolyte. For example, the hydrodynamic diameter of poly(NIPAM-co-AA) microgels decreases with the increase in the NaCl concentration (Figure 1-6)\textsuperscript{36}. This is attributed to the electrolyte screening on the internal charge repulsion, which is reversible by removing the electrolyte. It should be noted that the temperature may influence the response to changes in ionic strength when the microgels contain both thermo-sensitive and ionizable components. For example, in the presence of a low
concentration of electrolyte (such as NaCl), dilute P(NIPAM-co-AA) microgel dispersions are colloidally stable even when the temperature is above the VPTT of the microgels. However, at high ionic strengths, aggregation of microgels may occur, especially when the dispersion temperature of microgel particles is above their VPTT.

1.3.3.4 Other Influences

Other influences or triggers for inducing the swelling/shrinking transition of microgels include alcohol additions, light, and magnetism. For example, the swelling degree of PNIPAM microgel particles can be controlled by addition of short-chain alcohols such as methanol (MeOH), ethanol (EtOH) and 2-propanol (2-PrOH). As shown in Figure 1–7, the microgel particles collapse to a minimum size when volume fraction of alcohol increase, but re-swell in the alcohol-rich region.

![Figure 1-7](image)

**Figure 1–7** Change in the de-swelling ratio of PNIPAM (9% MBA) microgel particles as a function of increasing volume fraction of three alcohols. The alcohols investigated are MeOH (filled diamond), EtOH (empty square) and 2-PrOH (filled circle).
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1.4 Characterization

Several techniques are used to characterize microgel particles. They include light scattering, fluorometry, UV-VIS spectrophotometry, TEM and confocal imaging technique. Dynamic light scattering (DLS) has been the most widely used technique to study the size, size distribution, and the volume phase transition behavior of microgels. A dilute dispersion of microgels appears transparent because the microgels are swollen in water at $T < VPTT$ and the contrast in refractive indices of the polymer and the solvent is small. At $T > VPTT$, the expulsion of water from the particles causes an increase in refractive index contrast between the polymer and the solvent, and the microgel dispersion may appear turbid, depending on the concentration. TEM could show the morphology of dried microgel particles. More details of this experimental technique are provided in Chapter 2.

1.5 Applications of Microgels

The reversible shrinking/swelling transition of microgels in response to specific environmental conditions enables these “smart nanoparticles” to have great potential for a variety of applications. Indeed, they have already been utilized in a wide range of industries, including chemical sensing, drug delivery, and biomedical applications.

1.5.1 Biomaterials/Biomedical Applications

Microgels have been widely utilized in biomaterials area, including implantation, incorporation with a living system to supplement, and replace functions of living tissues\(^{39}\). The most fundamental requirement of biomaterials is the biocompatibility, which means that it
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should be able to co-exist with its tissue environment without having any undesirable or inappropriate effect on each other\textsuperscript{40}.

Cell Scaffolds

Zhang’s group reported that the \textit{in situ} gelable dispersions of poly(NIPAM-co-2-hydroxyethyl methacrylate) and poly(NIPAM-co-AA) microgels in electrolyte solutions of high ionic strength can be used as scaffolds for cell culture\textsuperscript{41–43}. Our group demonstrated that the cell scaffolds can be also synthesized from a colloidal supra-structures constructed by the thermo-driven gelation of a colloidal dispersion of poly(NIPAM-co-AAm) microgels (Figure 1–8)\textsuperscript{44}. The microgels exhibit a reversible and continuous volume transition in water with $VPTT = 35 \, ^\circ\mathrm{C}$ and remain partially swollen and soft under physiological conditions ($37 \, ^\circ\mathrm{C}$). More importantly, we found that the size of the microgel particles can affect the sol-to-gel phase transition of the microgel dispersions, alter the syneresis degree of the constructed colloidal supra-structures, and tailor the cytocompatibility when the colloidal supra-structures are used for cell scaffold. We believe the relationship between the size of the building microgel particles and the cytocompatibility of the constructed colloidal supra-structure could provide fundamental guidance for the construction of desirable scaffolds to regulate the cell proliferation rate.
The size of soft microgels can affect the thermo-driven sol-to-gel transition, the syneresis degree, and the cell proliferation rate of the constructed colloidal supra-structures for cell scaffolds\textsuperscript{44}.

**Detachment and Delivery of Human Cells**

Hopkins *et al.* modified poly(NIPAM) microgels with a cell-adhesive peptide (GRGDS), which bound to cell surface integrins on dermal fibroblasts and entholial cells\textsuperscript{45}. The microgels remove the cells from their normal culture substrate at temperature above LCST, and released the viable cells to grow on new substrates on cooling. The authors suggested that such systems will have a wide range of applications in cell biology and tissue engineering.

**Slimming Aids**

Microgels have been also applied as slimming aids\textsuperscript{46}. Microgels were embedded with a cationic fat-binding agent in an edible matrix, which disintegrates in the digestive tract. The microgels swell up to 100 times the dry volume in stomach, creating a feeling of fullness. Additionally, this fat-binding agent scavenges fatty acids, resulting in reduced absorption in the intestine.
1. 5. 2 Optical Sensor

Real-time measurements of biological/chemical/physical processes in live cells, with no interferences, are an ultimate goal for *in vivo* intracellular studies. Significant progress has been achieved by the continuous development of analyte-specific fluorescent probes, such as, organic dyes, semiconductor quantum dots and noble metal nanoparticles. However, the cytotoxicity of some available probes is a big problem and these moieties may affect immunological response of cells. Besides, non-specific binding and short lifetime of these molecular probes have to be considered as well. To overcome these problems, the integration of small molecule probes into other matrices such as polymers microgels could be a suitable choice. First, this matrix can minimize the toxicity of probes by protecting the cellular contents from the probes. The cell viability after sensor delivery can reach 99%, relative to control cells, indicating negligible physical and chemical perturbation to the cell. Second, microgels show some physical properties similar to living tissues, including a soft and rubbery consistency and low interfacial tension with water or biological fluids. The elastic nature of hydrated gels has been found to minimize irritation to the surrounding tissues after implantation. The low interfacial tension between the gel surface and body fluid minimizes protein adsorption and cell adhesion, which reduces the chances of a negative immune reaction. The inert protective matrix of the gel networks also eliminates interferences such as protein binding and/or membrane/organelle sequestration. Third, the specific functional groups on the microgel network chains enable the immobilization of optical moieties into the polymer gel networks, combining the properties from both optical moieties and polymer gels\(^{47}\). For example, the surface functionalization with targeting ligands on the environmentally responsive micro-/nanogels could target the delivery of optical probes to cells, in other word, selectivity and sensitivity of small molecular probes can be improved\(^{48}\).
Generally, a hybrid micro-/nanogel-based optical probe is composed of two components: optical moieties and a three-dimensional polymer scaffold. Under this consideration, such hybrid micro-/nanogel probes can be classified into three types (Figure 1–9)\(^49\).

**Figure 1–9** Schematic diagrams showing three types of hybrid micro-/nanogel-based optical probes\(^49\): (A) Type 1 where the antibody or specific targeting ligand acts as a chemical/biochemical signal receiver; (B) Type 2 where an optical moiety acts directly as the chemical / biochemical signal receiver; and (C) Type 3 where a responsive polymer gel network chain acts as the chemical/biochemical signal receiver, which will undergo a volume phase transition, change the physicochemical environment of the optical moieties, and convert the received signal into an optical signal.

In a Type 1 probe, the specific antibodies, aptamers, or other selective analyte-recognition ligands are covalently linked to the hybrid micro-/nanogels. Binding of the analyte to
the hybrid micro-/nanogels would not affect the optical properties of the optical moieties, but the apparent color may change upon colocalization of the probes. In a Type 2 probe, the optical moiety acts directly as the chemical/biochemical signal receiver. The optical properties of the optical moieties (such as indicator dye and conjugated polymer) would change upon the binding of analyte. In a Type 3 probe the stimuli-responsive polymer gel network chains act as the chemical/biochemical signal receiver. Upon receiving the external stimulus, the gel network will undergo a volume phase transition and change the physicochemical environment of the optical moieties, converting the received signal into optical signal.

Temperature Sensor

Our group has synthesized a group of core-shell structure hybrid nanogels to achieve optical temperature sensing. The nanogels were constructed by coating the Ag-Au bimetallic NP core with a thermo-responsive nonlinear poly(ethylene glycol) (PEG)-based hydrogel as shell. The Ag-Au NP core can emit strong visible fluorescence. The reversible thermo-responsive volume phase transition of the nonlinear PEG-based gel shell can modify the physicochemical environment of the Ag-Au NP core to manipulate the fluorescence intensity for sensing the environmental temperature change (Figure 1–10).

Figure 1–10 Schematic illustration of multifunctional core-shell hybrid nanogels.
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**Ions Sensor**

The polyacrylamide nanoparticle has been used exclusively for this type of ion sensor because of its neutral and hydrophilic nature, which allows ions to readily permeate polymer matrix and interact with the indicator dye. Sensors for cobalt, copper, hydrogen, nickel, potassium, silver, sodium, zinc, and chloride ions have been developed\(^{30}\).

**pH Sensor**

A pH sensor was developed by coating a pH-insensitive fluorescent polystyrene bead (200 nm in diameter) with a layer of polyaniline (PANI) of only a few nanometers thick\(^{51}\). Plain PANI films display no fluorescence in the visible and near-IR range, but they display characteristic pH-dependent absorption spectra that are due to protonation and deprotonation, respectively, of the emeraldine form of the PANI. Because of the fluorescence spectra of the beads being overlapped with the absorption spectra of PANI, the fluorescence intensity changes in accordance with the changes in pH.

Our group also report a class of chitosan-based hybrid nanogels by in-situ immobilization of CdSe quantum dots (QDs) in the chitosan-poly(methacrylic acid) networks. The covalently crosslinked hybrid nanogels with chitosan chains semi-interpenetrating in the crosslinked poly(methacrylic acid) networks exhibit excellent colloidal and structural stability as well as effectively sense the change of environmental pH\(^{52}\).
Glucose Sensor

Figure 1–11 Reversible fluorescence quenching and antiquenching of CdS QDs embedded in the interior of p(NIPAM-AAm-PBA) microgels in response to the change in glucose concentration. For the application in glucose detection, CdS QDs immobilized PNIPAM-based glucose-sensitive microgels have been designed as optical glucose sensor by our group (Figure 1–11). The photoluminescence (PL) intensity of CdS QDs can be quenched gradually when microgels gradually swelled up with the addition of D-glucose. Therefore, such hybrid microgels exhibit excellent sensitivity in physiologically important glucose concentration range (1–25 mM). Wu et al. in our group designed hybrid nanogels made of Ag NP cores covered by a copolymer gel shell of poly(4-vinylphenylboronic acid-co-2-(dimethylamino)ethyl acrylate) as well. These hybrid microgels can also be used as optical glucose sensor and show regulate release of preloaded insulin at physiological pH.

1.5.3 Intelligent Drug Delivery Systems

An increasing field of research is the development of devices that can control the release of rapidly metabolized drugs in order to protect sensitive drugs. Conventional delivery systems
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suffer from limitations of dose dumping, for example, the delivery of high dose of therapeutic in one go. Therefore, it is necessary to explore a new delivery system which can achieve an elevated therapeutic drug plasma concentration over a long period of time, known as controlled release. Microgels are particularly attractive as drug delivery carriers with several advantages: tunable size from 100 nm to several micrometers, their porous interior network structure for the incorporation of drugs to protect the drug from hydrolysis, potential biocompatibility, and functionalized surface with receptor-specific molecules to achieve targeting ability. In addition, microgels carrying specific functional groups can undergo large swelling-deswelling transitions in response to changes in pH, ionic strength, or temperature, which commonly occur in many biological events.

Figure 1–12 Model of triggerable drug release from the responsive microgels or shell-responsive core-shell structure microgels with drug in the core.

Generally, two systems could be applied in drug delivery (Figure 1–12). The first one is randomly functionalized microgels. Drug loaded microgels are able to undergo shrinkage or swelling when the environment is changed, which can trigger the release of the drug. The second one is core-shell structured microgels. In this case, the responsive shell of microgels act as a switch and drugs are incorporated within the core. As the change of environment occurs, the
responsive shell starts to swell along with the increased pore size; hence, drugs can diffuse to exterior.

Several microgel-based drug delivery systems have been investigated. For example, a co-polymer microgel of poly(NIPAM/butyl methacrylate/AA) has been applied as a delivery vehicle to carry insulin$^{55}$. Currently, insulin has to be injected intravenously because passage through a low pH of the stomach degrades insulin, reducing its activity. The hydrophobic butyl methacrylate facilitate the uptake of insulin into the interior of the microgel, and the incorporation of AA sensitizes the microgel to pH via protonation and de-potonation of the carboxylic acid groups above and below its $pK_a$ (pH 4.4). The microgels adopt a closed network structure below the $pK_a$ of AA, within which insulin can be entrapped. When pH rises above the $pK_a$, the conformation of microgel network chains changes to an open network structure because the intermolecular charge repulsion causes expansion. At this time, insulin is released from the open network structure via osmosis. This delivery system could be used for delivery of an oral therapeutic. The therapeutic can be protected from stomach acid (pH $\approx$ 2). When the drug-loaded microgels move out of the stomach into the gastrointestinal (GI) tract, they change conformation and release the therapeutic due to pH increase.

Our group has also contributed much effort to investigate the storage and delivery of chemotherapeutic agents using microgels as drug delivery carriers$^{56-58}$. For example, a series of water-dispersible core-shell structured microgels with a hydrophobic polystyrene (PS) gel as core and a hydrophilic nonlinear PEG copolymer gel as shell have been successfully synthesized for intracellular delivery of hydrophobic curcumin drug. While the PS nanogel core is designed to provide strong hydrophobic interactions with curcumin for high drug loading yields, the thermo-responsive nonlinear PEG gel shell is introduced to provide good water dispersibility of
the microgels and temperature triggerable release of the pre-loaded curcumin drug. A systematic physicochemical study on the resulted microgels was carried out to optimize the compositions and structural parameters of the microgels to achieve high curcumin loading capacity, responsiveness in physiologically important temperature range, and thermally controllable curcumin release. The curcumin molecules released from microgels still remain active to kill the cancer cells. Such designed core-shell structured microgel is well suited for drug uptake and release application and could be potentially extended to effectively deliver other hydrophobic therapeutic agents.

1.6 Conclusion

Stimulus-responsive polymer microgels swell and shrink reversibly upon exposure to various environmental stimuli such as change in pH, temperature, glucose concentration, ionic strength or magnetic fields. They are usually synthesized by precipitation polymerization. Due to the stimuli responsive properties, they become ideal candidates for biomaterial applications, optical sensors and imaging agents, and primary carriers in site-specific and controlled drug delivery systems. Facile synthesis and functionalization of microgel particles provide a broad range of variables for tuning their properties and favorably distinguish microgels from other particulate polymer materials used for similar applications.
Chapter 1

1.7 References


Chapter 1


Chapter 1


Chapter 1


Chapter 2

Materials and Methods

This chapter presents the basic synthetic and characterization methods used in this dissertation projects. The standard synthetic method for preparation of microgels by free radical precipitation polymerization is discussed. The typical method for preparation and characterization of inorganic nanoparticles that can be immobilized into the micro-/nanogels are also provided.

2.1 Preparation of Microgels

2.1.1 Reagents

D(+)-Glucose was purchased from ACROS and all other chemicals were purchased from Sigma-Aldrich. N-isopropylacrylamide (NIPAM) was purified by recrystallization from a hexane–acetone (1 : 1 volume ratio) mixture and dried in a vacuum. 2-(2-methoxyethoxy)ethyl methacrylate (MEO₂MA, 95%), oligo(ethylene glycol)methyl ether methacrylate (Mₙ = 300 g/mol, MEO₃MA), 2-(Dimethylamino)ethyl acrylate (DMAEA) and poly(ethylene glycol) dimethacrylate (PEGDMA, Mₙ ≈ 550 g/mol, crosslinker) were purified with neutral Al₂O₃.
Curcumin was purified with anhydrous ethanol. 2-vinylanisole, 4-allylanisole, 4-vinylphenylboronic acid (VPBA), divinylbenzene (DVB), N,N´-methylenesbis(acrylamide) (MBAAm), 2,2’-azobis(2-methylpropionamidine) dihydrochloride (AAPH), ammonium persulfate (APS), sodium dodecylsulfate (SDS), anhydrous ethanol, dimethyl sulfoxide (DMSO), acridine orange (AO), ethidium bromide (EB), dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), and the lyophilized fluorescein isothiocyanate-labeled insulin (FITC-insulin) from bovine pancreas (~ 5800 Da) were used as received without further purification. The water used in all experiments was of Millipore Milli-Q grade. Table 2–1 shows the structure and functions of the reactants used for microgels synthesis in this work.
Table 2-1 Structures and function of reactants used in free radical precipitation polymerization for the synthesis of microgels in this work.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure" /></td>
<td>N-isopropylacrylamide (NIPAM)</td>
<td>Major monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thermo-responsive component</td>
</tr>
<tr>
<td></td>
<td>Acrylamide (AAm)</td>
<td>Comonomer</td>
</tr>
<tr>
<td><img src="image2" alt="Structure" /></td>
<td>2-Vinylanisole</td>
<td>Major monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curcumin loading component</td>
</tr>
<tr>
<td><img src="image3" alt="Structure" /></td>
<td>4- Allylanisole</td>
<td>Major monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curcumin loading component</td>
</tr>
<tr>
<td><img src="image4" alt="Structure" /></td>
<td>2-(2-methoxyethoxy)ethyl methacrylate (MEO₂MA)</td>
<td>Major monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thermo-responsive component</td>
</tr>
<tr>
<td><img src="image5" alt="Structure" /></td>
<td>Oligo(ethylene glycol)methyl ether ethacrylate (MEO₃MA)</td>
<td>Major monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thermo-responsive component</td>
</tr>
<tr>
<td><img src="image6" alt="Structure" /></td>
<td>4-Vinylphenylboronic acid (VPBA)</td>
<td>Major monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose-responsive component</td>
</tr>
<tr>
<td><img src="image7" alt="Structure" /></td>
<td>2-(Dimethylamino)ethyl acrylate (DMAEA)</td>
<td>Comonomer</td>
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<tr>
<td><img src="image8" alt="Structure" /></td>
<td>N,N’-Methylenebis(acrylamide) (MBAAm)</td>
<td>Crosslinker</td>
</tr>
<tr>
<td><img src="image9" alt="Structure" /></td>
<td>Divinylbenzene (DVB)</td>
<td>Crosslinker</td>
</tr>
<tr>
<td><img src="image10" alt="Structure" /></td>
<td>Poly(ethylene glycol) dimethacrylate (PEGDMA)</td>
<td>Crosslinker</td>
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<tr>
<td><img src="image11" alt="Structure" /></td>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Surfactant</td>
</tr>
<tr>
<td><img src="image12" alt="Structure" /></td>
<td>Ammonium persulfate (APS)</td>
<td>Initiator</td>
</tr>
<tr>
<td><img src="image13" alt="Structure" /></td>
<td>2,2’-azobis(2-methylpropionamidine) dihydrochloride (AAPH)</td>
<td>Initiator</td>
</tr>
</tbody>
</table>
2.1.2 Synthesis of Microgels

The microgels in this work were synthesized by free radical precipitation polymerization.\(^1\) This method is probably the most frequently used technique for microgel synthesis. The mechanism of free radical precipitation polymerization has been introduced in Chapter 1 (Section 1.2.2). Here, we describe a typical example using the precipitation polymerization technique for preparation of poly(NIPAM-\textit{co}-AAm) microgels in this work. Typically, NIPAM (1.405 g), AAm (0.091 g), MBAAm, and SDS were dissolved in 100 mL deionized water in a 250 mL round-bottom flask equipped with a stirrer, a \(\text{N}_2\) gas inlet, and a condenser. The mixture was heated to 70 °C under a \(\text{N}_2\) purge. After 1 h, APS (0.104 g dissolved in 5.0 mL water) was added to the mixture to initiate the polymerization. The reaction was allowed to proceed for 5 h. The obtained products were purified by centrifugation (Thermo Electron Co. SORVALL® RC-6 PLUS superspeed centrifuge, 35 °C, 20 000 rpm, and 20 min) and 3 days of dialysis (Spectra/Por® molecular porous membrane tubing, cut off 12 000–14 000) against very frequently changed water at room temperature (\(\sim 22 \, ^\circ \text{C}\)).

2.1.3 Purification of Microgels

The solutions might contain surfactant molecules and a certain amount of linear or slightly branched polymer segments after polymerization. Generally, there are two ways to purify the microgels. First, effectively repeated centrifugation, decantation, and redispersion of the microgels in water can be applied. Depending on the property of microgels, centrifugation conditions may be varied (revolution, temperature, pH, and \textit{etc.}). Second, unreacted monomers and excess surfactant can be removed by dialysis against frequently changes of water for a long enough time. However, dialysis even over extended periods of time is not always sufficient to
remove all linear polymers or sol from microgel dispersions, a combination of dialysis and repeated centrifugation, decantation and redispersion techniques under appropriate conditions can effectively remove linear polymers from a dispersion of microgels. In the present work, microgels were typically purified by first centrifugation and redispersion, and then followed by dialysis against frequently changed deionized water for 3–5 days.

2. 2 Characterization of microgels

2. 2. 1 Transmission electron microscopy

The morphology of microgels was characterized with transmission electron microscopy (TEM). Detailed information on the theory of TEM can be found elsewhere. Briefly, a beam of electrons are focused on a single, pinpoint spot or element on the sample being studied. The electrons interact with the sample and only those that go past unobstructed hit the phosphor screen on the other side. At this point, the electrons are converted to light and an image is formed. The basic set up for TEM is schematically shown in Figure 2–1. Approximately 10 µL of diluted microgel suspension in water was dropped on a carbon-coated copper grid and then air-dried at room temperature. The TEM images of the dried samples were taken on a FEI TECNAI transmission electron microscope operating at an accelerating voltage of 120 kV.
Figure 2–1 Schematic illustration of Transmission Electron Microscope.

The dark areas of the image correspond to areas on the specimen where fewer electrons are able to pass through (they are either absorbed or scattered upon impact); the lighter areas are where more electrons pass through, although the varying amounts of electrons in these areas enable the user to see structures and gradients.

The 'lenses' in a TEM are not the same as lenses in a conventional microscope. These are actually the EM devices that can 'focus' the electron beam to the desired wavelength or size. In much the same way as a light microscope, however, the amount of power used to generate electrons allows for higher magnification or better resolutions\(^2\).
2.2.2 Size and Size Distribution

A standard light scattering spectrometer (BI-200SM) equipped with a BI-9000 AT digital
time correlator (Brookhaven Instrument, Inc.) was used to monitor the size and size distribution
of the microgels under different conditions. The schematic layout of the instrument is shown in
Figure 2–2. A Nd: YAG laser (150 mW, 532 nm) was used as the light source. The solutions
were passed through 0.8 µm Millipore Millex-HV filters to remove dust. In Dynamic light
scattering, the Laplace inversion of each measured intensity–intensity time correlated function
can result in a characteristic line width distribution \( G(\Gamma) \). For a purely diffusive relaxation, \( \Gamma \) is
related to the translational diffusion coefficient \( D \) by \( (\Gamma/q^2)_{C \rightarrow 0, q \rightarrow 0} = D \), where \( q =
(4\pi n/\lambda)\sin(\theta/2) \), with \( n, \lambda \), and \( \theta \) being the solvent refractive index, the wavelength of the incident
light in vacuum, and the scattering angle, respectively. \( G(\Gamma) \) can be further converted to a
hydrodynamic radius \( (R_h) \) distribution by using the Stokes–Einstein equation,
\( R_h = (k_BT/6\pi\eta)D^{-1} \),
where \( T, k_B, \) and \( \eta \) are the absolute temperature, the Boltzmann constant, and the solvent
viscosity, respectively.

\[ \text{Figure 2–2 Schematic diagram of a conventional light scattering instrument setup.} \]
2. 2. 3 Confocal Microscopes

The cells incorporated with microgels were imaged using a confocal laser scanning microscopy (LEICA TCS SP2 APBS™) equipped with an HC PL APO CS 20 × 0.7 DRY len. A UV (405 nm) light was used as the light source. The schematic layout of the instrument is shown in Figure 2–3.

![Figure 2–3 Schematic of confocal microscope.](image)

A laser is used to provide the excitation light (in order to get very high intensities). The laser light (blue) reflects off a dichroic mirror. From there, the laser hits two mirrors which are mounted on motors; these mirrors scan the laser across the sample. The labeling molecules (e.g., dye, quantum dots) in the sample fluoresce, and the emitted light (green) gets descanned by the same mirrors that are used to scan the excitation light (blue) from the laser. The emitted light
passes through the dichroic and is focused onto the pinhole. The light that passes through the pinhole is measured by a detector.

2.2.4 Cell toxicity

We used MTT assay to quantitatively evaluate the cell viability. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazole. The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple color. A soluble solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a Nikon fluorescence microscope fitted with a Spot digital camera. The absorption maximum is dependent on the solvent employed.

Other closely related tetrazolium dyes including XTT, MTS and the WSTs, are used in conjunction with the intermediate electron acceptor, 1-methoxy PMS. With WST-1, which is cell-impermeable, reduction occurs outside the cell via plasma membrane electron transport. These assays measure cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and may, under defined conditions, reflect the number of viable cells (cell proliferation). Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferative to resting status) of potential medicinal agents and toxic materials.
Chapter 2

2.3 Synthesis of inorganic nanoparticles (NPs) in the microgels

2.3.1 Synthesis of Ag NPs

Citrate-stabilized Ag NPs were first prepared by dropwise addition of fresh NaBH₄ solution (10.6 mM, 2.5 mL) to an aqueous solution of AgNO₃ (0.1 mM, 200 mL) in the presence of sodium citrate (0.1 mM) under vigorous stirring. The resultant solution was stirred for 1 h and aged for 7 days at ambient conditions before use. The long aging time is necessary for completely degrading the reducing agent of NaBH₄. SDS stabilized Ag NPs were obtained by adding 0.053 g SDS into 100 mL of aqueous solution of citrate-stabilized Ag NPs in a 250 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, and then aging the mixture for 10 h⁸.

2.3.2 Synthesis of Fe₃O₄ NPs

In a 25 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, 5 mmol of FeCl₃, 2.5 mmol of FeCl₂·4H₂O, and 30 mL deionized water was heated to 80 °C, followed by addition of NaOH (5 mL, 10M) under stirring. This reaction was allowed to perform for 1 hour. Then the resulted solution was centrifuged three times at 6000 rpm (20 min, Thermo Electron Co. SORVALL RC-6 PLUS superspeed centrifuge) with the supernatant discarded and the precipitate was redispersed in 30 mL deionized water. SDS-stabilized Fe₃O₄ NPs were obtained by adding 0.1322 g SDS into 100 mL deionized water containing 0.125 mL Fe₃O₄ NPs in a 250 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, and then aging the mixture for 10 h⁹.
2. 3. 3 Characterizations of NPs

The obtained Ag NPs in this work were characterized by TEM and spectrophotometers for both absorption and emission properties. The Fe$_3$O$_4$ NPs were characterized by TEM and a superconducting quantum interference device (SQUID) magnetometer (Quantum Design MPMS XL-7) to measure the field dependent magnetization.
2.4 References


Chapter 3

A Colloidal Supra-structure of Responsive Microgels as a Potential Cell Scaffold

3.1 Introduction

After significant advances in the synthesis and modification of a wide variety of colloidal particles at the micro-/nanoscale, the scientific community has seen increasing interest in the construction of complex three-dimensional (3D) supra-structures with these colloidal particles as building blocks. The distinct properties of the colloidal particles can be harnessed in suprastructures with new collective properties and fascinating applications in optics, electronics, magnetic storage, and biology. In many aspects, colloidal particles dispersed in a liquid behave similarly to large idealized atoms that exhibit both fluid-like and solid-like phases, with the transition between the two phases taking different forms. Phase transitions in colloidal particle dispersions have been intensively studied as a result of their utility as models for addressing fundamental questions about the nature of molecular and atomic condensed phases. A wide variety of colloidal supra-structures have been generated by using hard and quasi-hard
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particles such as Ag, polystyrene, and polymethylacrylate\textsuperscript{1–3, 9, 14}. However, relatively little attention has been paid to those soft particles which can respond to environmental stimuli like temperature.

Injectable macroscopic gels (macrogels) are highly desirable biomaterials for tissue engineering to repair or regenerate damaged and diseased tissues or serving as in vitro 3D models for drug screening and tumor studies\textsuperscript{15–17}. Up to now, nearly all thermal gelling injectable scaffolds employ linear or branched polymers. On the other hand, the in situ formation of macrogels from the assembly of temperature-responsive microgel particles into colloidal suprastructures could be a simple and effective alternative approach because precise control of the composition, size and size distribution, and volume phase transition temperature (VPTT) of microgels is relatively easy to achieve\textsuperscript{18–21}. Potential advantages of microgel particles over linear or branched polymers include reduced viscosity and better mechanical properties at the same concentration. The use of microgel particles may also open the possibility to encapsulate growth factors or other bioactive molecules in the interior of the particles and release them in proper time to guide the differentiation of cells\textsuperscript{15–17}. The thermo-driven gelling behavior of poly(N-isopropylacrylamide) (polyNIPAM)-based microgel dispersions in NaCl solution or at high concentrations were reported about a decade ago\textsuperscript{22, 23}. However, to the best of our knowledge, only recently, Zhang’s group reported that the in situ gelable dispersions of poly(N-isopropylacrylamide-co-2-hydroxyethyl methacrylate) and poly(N-isopropylacrylamide-co-acrylic acid) microgels in electrolyte solutions of high ionic strength can be used as scaffolds for cell culture\textsuperscript{24–26}. In a recent literature reported by Schmidt et al.\textsuperscript{27}, polyNIPAM microgel layers were used as smart substrates for cell culture. It was proposed that the Young’s modulus of the individual microgels is a key parameter for the interaction with mammalian cells.
In this work, we studied the thermo-driven in situ gelation of poly(N-isopropylacrylamide-co-acrylamide) (poly(NIPAM-co-AAm)) microgel particles for potential cell scaffolds (Scheme 3–1). Different from the previous examples in which the VPTTs of the poly-NIPAM-based microgels are much below 37 °C\textsuperscript{22–26}, making them fully collapsed and possibly hard-sphere-like under physiological conditions, the presented poly(NIPAM-co-AAm) microgels with a VPTT $\approx$ 35 °C in water are partially swollen and soft under physiological conditions. The constructed colloidal supra-structures from these poly(NIPAM-co-AAm) microgels can be regarded as a model system for a new class of cell scaffolds. According to percolation theory\textsuperscript{28}, the microgel particle arrangements in the supra-structure should depend on the fraction occupied by the microgel particles. As a proof-of-concept, we show that the size of microgel particles, a key parameter associated with the particle volume, could affect the thermo-driven gelling behavior of microgel dispersions, alter the syneresis degree of the formed colloidal supra-structures, and tailor the cytocompatibility of the resulted colloidal suprastructures for cell scaffolds. The relationship between the size of the building microgel particles and the cytocompatibility of the constructed colloidal supra-structures could provide fundamental guidance for the construction of desirable cell scaffolds to regulate the cell proliferation rate\textsuperscript{16}, a key issue that has not been addressed in previous work.
The size of soft microgels can affect the thermo-driven sol-to-gel transition, the syneresis degree, and the cell proliferation rate of the constructed colloidal supra-structures for cell scaffolds.

### 3.2 Experimental

#### 3.2.1 Materials

All chemicals were purchased from Sigma-Aldrich. N-isopropylacrylamide (NIPAM) was purified by recrystallization from a hexane–acetone (1 : 1 volume ratio) mixture and dried in a vacuum. N,N’-Methylenebis(acrylamide) (MBAAm), acrylamide (AAm), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), acridine orange (AO), ethidium bromide (EB), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were used as received without further purification. The water used in all experiments was of Millipore Milli-Q grade.
3.2.2 Synthesis of poly(NIPAM-co-AAm) microgels

A series of poly(NIPAM-co-AAm) microgels were synthesized by using free radical precipitation polymerization in an aqueous solution. Typically, NIPAM (1.405 g), AAm (0.091 g), MBAAm, and SDS were dissolved in 100 mL deionized water in a 250 mL round-bottom flask equipped with a stirrer, a N\textsubscript{2} gas inlet, and a condenser. The mixture was heated to 70 °C under a N\textsubscript{2} purge. After 1 h, APS (0.104 g dissolved in 5.0 mL water) was added to the mixture to initiate the polymerization. The reaction was allowed to proceed for 5 h. The obtained products were purified by centrifugation (Thermo Electron Co. SORVALL\textsuperscript{®} RC-6 PLUS superspeed centrifuge, 35 °C, 20 000 rpm, and 20 min) and 3 days of dialysis (Spectra/Por\textsuperscript{®} molecular porous membrane tubing, cut off 12 000–14 000) against very frequently changed water at room temperature (∼22 °C).

3.2.3 TEM characterization

Approximately 10 µL of diluted microgel suspension in water was dropped on a carbon-coated copper grid and then air-dried at room temperature. The transmission electron microscopy (TEM) images of the dried samples were taken on a FEI TECNAI transmission electron microscope operating at an accelerating voltage of 120 kV.

3.2.4 Dynamic light scattering (DLS) characterization

A standard laser light scattering spectrometer (BI-200SM) equipped with a BI-9000 AT digital time correlator (Brookhaven Instruments, Inc.) was used, with a Nd:YAG laser (150 mW, 532 nm) as the light source. The extremely diluted microgel dispersion (∼5.0 \times 10^{-3} wt\%) in 0.005 M phosphate buffered saline (PBS) of pH = 7.4 was passed through Millipore Millex-HV
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filters with a pore size of 0.80 \( \mu m \) to remove dust before DLS measurements. In DLS, the Laplace inversion of each measured intensity–intensity time correlated function can result in a characteristic line width distribution \( G(\Gamma) \). For a purely diffusive relaxation, \( G \) is related to the translational diffusion coefficient \( D \) by \( (\Gamma/q^2)_{c \to 0, q \to 0} = D \), where \( q = (4\pi n/\lambda)\sin(\theta/2) \) with \( n, \lambda, \) and \( \theta \) being the solvent refractive index, the wavelength of the incident light \( \text{in vacuo} \), and the scattering angle, respectively. \( G(\Gamma) \) can be further converted to a hydrodynamic radius (\( R_h \)) distribution by using the Stokes–Einstein equation, \( R_h = (k_B T/6\pi \eta \bar{D})^{-1} \), where \( k_B, T, \) and \( \eta \) are the absolute temperature, the Boltzmann constant, and the solvent viscosity, respectively\(^{29,30}\).

3.2.5 \( \zeta \)-Potential characterization

Experiments of electrophoretic mobility \( \mu_E \) were carried out on a Malvern Zetasizer Nano S90 particle analyzer on the Zeta-Meter mode and calibrated with standard solutions. The \( \zeta \)-potential of the microgel particles was calculated from the Smoluchowski relationship\(^{31}\):

\[
\zeta = \frac{\mu_E \eta}{\varepsilon_0 \varepsilon_r}
\]

(3–1)

where \( \varepsilon_0 \) is the permittivity of a vacuum, \( \varepsilon_r \) is the relative dielectric permittivity of the medium, and \( \eta \) is the viscosity of the dispersing phase.

3.2.6 Rheological characterization

The purified microgel dispersion was centrifuged and redispersed into water to prepare the concentrated microgel dispersions of different concentrations. Dynamic rheological analysis of the concentrated microgel dispersions was performed on an AR2000ex 45 rheometer (TA Instruments Ltd.). An aluminum parallel plate geometry with a diameter of 40 mm was used. The
sample gap was set to 1.0 mm. The temperature was controlled by using a water bath via connecting the bottom plate to a NESLAB RTE 7 circulating water bath circulator (Thermo Electron Co.). The temperature-dependent elastic (storage) modulus, $G'$, and viscous (loss) modulus, $G''$ were recorded in a dynamic temperature ramp test (DTRT). The DTRT was conducted under a controlled stress of 0.05 Pa, frequency of 0.1 Hz, and temperature ramp rate of $1 \, ^\circ\text{C} \, \text{min}^{-1}$ from 10 $^\circ\text{C}$ to 50 $^\circ\text{C}$. All the rheological experiments were performed within the linear viscoelastic region.

### 3.2.7 Syneresis

The syneresis kinetics were studied by using a modified procedure on the basis of a literature method reported previously\textsuperscript{26, 32}. Typically, equal amounts of 4.0 wt% microgel dispersion were added to small glass tubes with a diameter of 5 mm. They were brought to 37.0 $^\circ\text{C}$ to form the colloidal supra-structures (macrogels). Then the change in the macrogel length with time ($L_t$) was followed and measured with the assistance of a Nikon optical microscope.

### 3.2.8 Cell culture

B16F10 cell dispersion with a concentration of $2 \times 10^5$ cells per mL was mixed with an equal volume of 4.0 wt% microgel dispersion. To each well of a 48-well culture plate, 1 mL of the cell–microgel mixture was added. The mixtures were brought to 37 $^\circ\text{C}$ and gelated immediately. After 2 h, the cell/scaffold constructs were transferred to culture flasks and cultured in a media containing 89% Dulbecco’s Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cultures were maintained in an incubator at 37 $^\circ\text{C}$ with a humidified atmosphere of 5% CO\textsubscript{2}. The medium was changed every 1–3 days.
3. 2. 9 Cell viability

A live/dead assay was performed to evaluate the cell viability after 1, 4, 7, 14, and 21 days of cell seeding. Briefly, 0.5 mL of cell/scaffold constructs were transferred to 6-well culture plate with 3 mL of a media containing 89% DMEM, 10% FBS and 1% penicillin–streptomycin. The medium was changed every 1–3 days. The cultures were maintained in an incubator at 37 °C with a humidified atmosphere of 5% CO₂ for a predetermined period. Then, the samples were stained with AO and EB. An upright Nikon fluorescence microscope fitted with a Spot digital camera was used to observe the appearance of the cells.

3. 2. 10 Quantification of cell viability by using MTT assay

After cultured for a predetermined period (1, 4, 7, 14, and 21 days), the medium was aspirated and these wells were washed using fresh serum-free DMEM. Then, 100 mL of MTT solution (5 mg mL⁻¹ in PBS) were added to the wells. After incubation for 2 h, the solution was aspirated. 500 mL of DMSO was then added to each well and the plate was sealed and incubated for 30 min at 37 °C with gentle mixing. Three portions of the solution obtained from each well were transferred to three respective wells of a 96-well plate (100 mL per well). Cell viability was measured using a microplate reader at 490 nm. Positive controls contained no microgels, and negative controls contained MTT. Parallel wells also contained only medium (no cells) and the same concentrations of microgels.
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3. 3 Results and discussion

3. 3. 1 Synthesis of poly(NIPAM-co-AAm) microgels

![Figure 3–1 Typical TEM image of the poly(NIPAM-co-AAm) microgels.](image)

The temperature-responsive poly(NIPAM-co-AAm) microgels were synthesized from the free radical precipitation copolymerization of NIPAM and AAm using MBAAm as a crosslinker. The reactivity ratio of NIPAM and AAm had been narrowly defined with the $Q$ and $e$ values being $Q = 0.21$, $e = 0.43$ for the former, and $Q = 0.23$, $e = 0.54$ for the latter, respectively\(^{33}\). With the similar monomer reactivity, the resulted poly(NIPAM-co-AAm) copolymer microgels should have randomly distributed polyNIPAM and polyAAm domains through the copolymer chains. It has been reported by our group and other groups that the contents of functional monomer AAm in poly(N-isopropylacrylamide-co-acrylic acid-co-acrylamide) microgels and other polyNIPAM-based microgels are nearly equal to their feeding compositions\(^{19, 34}\). As shown in Figure 3–1, the resultant poly-(NIPAM-co-AAm) microgel particles have a spherical morphology with a very narrow size distribution. The size of the
poly(NIPAM-co-AAm) microgel particles is tunable by changing the synthetic conditions. The increase in the concentration of dispersing surfactant SDS or crosslinker can significantly reduce the size of microgel particles (Table 3–1). All obtained microgel particles have a very narrow size distribution with a polydispersity index of $\mu^2/\langle \Gamma^2 \rangle \leq 0.005$ (Figure 3–2). The microgels are reproducible from batch to batch.

**Table 3–1** Dependence of microgel particle size ($R_h$) on feeding ratios of NIPAM/BIS/SDS. All measurements were made at 37.0 °C.

<table>
<thead>
<tr>
<th>Code</th>
<th>NIPAM/BIS/SDS (mol/mol/mol)</th>
<th>$R_h$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM1</td>
<td>100/2.57/2.57</td>
<td>110.4</td>
</tr>
<tr>
<td>NM2</td>
<td>100/1.68/2.57</td>
<td>120.5</td>
</tr>
<tr>
<td>NM3</td>
<td>100/2.57/1.54</td>
<td>143.7</td>
</tr>
<tr>
<td>NM4</td>
<td>100/2.57/0.51</td>
<td>190.1</td>
</tr>
<tr>
<td>NM5</td>
<td>100/2.57/0.26</td>
<td>230.4</td>
</tr>
</tbody>
</table>

**Figure 3–2** Size distribution of poly(NIPAM-co-AAm) microgels NM1 (■), NM2 (●), NM3 (▲), NM4 (▼), and NM5 (♦). All measurements were made at 37 °C and a scattering angle 45°.
3.3.2 Temperature-responsive volume phase transition and surface charge of poly(NIPAM-co-AAm) microgels

Figure 3–3 (A) Temperature-dependent average $R_h$ value of poly(NIPAM-co-AAm) microgels NM1 (■), NM2 (●), NM3 (▲), NM4 (▼), and NM5 (♦). (B) Size distributions of NM5 at different temperatures (♦: 24 °C; ▼: 33 °C; ▲: 37 °C; ●: 39 °C; and ■: 50 °C). All measurements were made at pH = 7.4 and a scattering angle $\theta = 45^\circ$. 

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Figure 3–3A shows the temperature-induced volume phase transition of the extremely
diluted poly(NIPAM-co-AAm) microgel dispersions (∼5.0 × 10⁻³ wt%) in 0.005 M PBS of pH =
7.4, in terms of the change of $R_h$ measured at a scattering angle of $\theta = 45^\circ$. It is clear that the
increase in temperature can lead to a significant decrease in the size of microgels due to the
presence of temperature-responsive polyNIPAM segments. The driving force for such a
temperature-induced volume phase transition is generally considered to be a subtle balance
between the ability of the polymer to form hydrogen bonds with water and the inter and
intramolecular hydrophobic forces\(^{35}\). In comparison with the pure polyNIPAM microgels that
present a relatively sharp decrease in $R_h$ at the VPTT of ∼32.5 °C\(^{36}\), all the poly(NIPAM-co-
AAm) copolymer microgels synthesized with the same AAm/NIPAM ratio of ∼0.11 exhibited a
similar volume phase transition profile and displayed two evident effects: (a) a VPTT shift to a
higher temperature of ∼35 °C; and (b) a broader volume phase transition temperature region.
Both effects are likely attributed to the presence of the hydrophilic polyAAm segments, which
can form extensive hydrogen bonds with water and lead to a higher VPTT. A gel network can be
visualized as a set of subnetworks\(^{37}\). The poly(NIPAM-co-AAm) copolymer microgels should
have randomly distributed polyNIPAM and polyAAm rich domains through the copolymer
chains. As the temperature increases, the sub-network chains with shorter polyNIPAM segments
will undergo the phase transition after the subnetworks with longer polyNIPAM segments. Thus,
different parts of the gel network undergo the phase transition at different temperatures.
Therefore, the volume phase transition of poly-(NIPAM-co-AAm) microgels should be
practically broadened because the subchains normally have a broad chain length distribution.
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Figure 3−4 ζ-potential of poly(NIPAM-co-AAm) microgels NM1 (■), NM2 (●), NM3 (▲), NM4 (▼), and NM5 (♦). All measurements were made at pH = 7.4.

It should be noted that the diluted poly(NIPAM-co-AAm) microgel dispersions (∼5.0 × 10^{-3} wt%) are highly stable without aggregation even at a temperature much higher than VPTT, as revealed by a single peak with the polydispersity index \( \mu_2/\langle I^2 \rangle \leq 0.005 \) being detected in DLS (Figure 3−3B). This may be attributed to the incorporation of the hydrophilic polyAAm segments, which can introduce the steric-hydration force, a short-range repulsion arising from the water structuring near hydrophilic surfaces. Water molecules are strongly bound to the surface of the hydrophilic amide groups of polyAAm segments. As two microgel particles approach each other, the repulsion arises because of the energy needed to confine and dehydrate the hydrophilic groups. Moreover, the use of initiator APS at high concentrations (e.g., the APS/NIPAM molar ratio of 0.28) in the synthesis would create ionizable sulfate groups (pKa < 2) on the surface of the microgel particles, which can further stabilize the microgels in water.
However, in our synthesis for poly(NIPAM-co-AAm) microgels, the APS/NIPAM molar ratio was only as low as 0.036. Although from a theoretical viewpoint the z-potential is electric potential in the interfacial double layer at the location of the slipping plane versus a point in the bulk solution away from the interface, the $\zeta$-potential profiles (Figure 3–4) indicated a nearly neutral surface for our microgels. The small amount of sulfate groups may have a negligible effect on the stability of our microgels. This is further confirmed by DLS analysis. It is well known that if the colloidal particles are charged, the electrostatic interactions between the colloidal particles can be screened by adding salt, leading to a poor stability of the microgels at a high ionic strength\(^{40}\). In our poly(NIPAM-co-AAm) microgel system, the size and size distribution of the microgels were nearly independent of the ionic strength in the range of 0.005–0.154 M. No aggregation of microgels was determined when heated. No cluster or suprastructure was formed in the diluted poly(NIPAM-co-AAm) microgel dispersions.

### 3.3.3 Formation of a colloidal supra-structure of poly(NIPAM-co-AAm) microgels

The temperature-responsive poly(NIPAM-co-AAm) microgel particle can serve as building block for the construction of a solid-like colloidal supra-structure. Such a supra-structure can be prepared by re-dispersion of the concentrated microgel particles into a 0.01 M PBS of pH = 7.4, whose ionic strength was subsequently adjusted to 0.154 M by adding NaCl to mimic physiological conditions, followed by heating to 37.0 °C. Figure 3–5 shows a typical dynamic stress sweep curve of a dispersion containing 4 wt% microgel particles (NM5: $\phi_{\text{effective}} = 0.04$) at 37.0 °C and a frequency of 0.1 Hz. The formation of the solid-like colloidal supra-structure can be readily demonstrated by the rheological characteristics that the storage modulus $G'$ is larger than the loss modulus $G''$ when the stress is below 0.1 Pa, where both $G'$ and $G''$ do
not change and thus can be defined as a linear viscoelastic region for this system. This solid-like colloidal suprastructure tends to respond strongly to an applied stress such that a solid-to-fluid transition occurs above a critical stress value. The stress-induced sharp decrease in dynamic modulus can be attributed to the sliding between the microgels\textsuperscript{41}, which is expected because the interaction between the microgels is a weak physical force. At a stress above ∼0.4 Pa, $G''$ is larger than $G'$, indicating that the system behaves in a liquid-like fashion. Interestingly, by simply cooling this liquid-like system to room temperature (∼25 °C) and then reheating to 37.0 °C, the solidlike colloidal supra-structure was formed again. The reproducibility of the supra-structure is clearly demonstrated by the good match of the dynamic stress sweep curves during the breakage/reconstruction cycles (Figure 3–5).

![Figure 3–5](image-url)

**Figure 3–5** Stress dependence of dynamic modulus ($G'$: ■, □; $G''$: ●, ○) of a 4 wt% poly(NIPAM-co-AAm) microgel dispersion in the presence (ionic strength 0.154 M; solid symbols) and absence (ionic strength 0.005 M; open symbols) of NaCl, respectively. All measurements were made at 37.0 °C and an oscillation frequency of 0.1 Hz.
To examine the in situ thermo-driven formation process of the solid-like colloidal supra-structure from the poly(NIPAM-co-AAm) microgel dispersion, a dynamic temperature ramp test has been carried out in the linear viscoelastic region (at a stress of 0.05 Pa and a frequency of 0.1 Hz) to record the changes of dynamic modulus as a function of temperature. Figure 3−6 shows that the sol-to-gel transition of the concentrated microgel dispersions upon heating can elegantly occur at pH = 7.4. At low temperatures, $G''$ is larger than $G'$, indicating that the dispersions are in the liquid-like state (sol). With an increase in temperature, both $G'$ and $G''$ first decrease, which is attributed to the shrinking of the microgel particles. The shrinking of the microgel particles (Figure 3−3) can lead to a decrease in volume fraction $\phi$, and thus a decrease in viscosity and elastic properties\(^{42}\). As temperature increases further, a sharp increase in both $G'$ and $G''$ occurs. A crossover of $G'$ and $G''$ was observed at the temperature of 35−36 °C. We define this temperature threshold for the formation of the solid-like colloidal supra-structure as the gelation temperature ($T_{gel}$). Above this temperature, $G'$ is generally larger than $G''$, indicating the formation of a particle network that is able to transmit stresses\(^{28}\), i.e. a transition from sol to a physical macrogel. All five microgel dispersions displayed a similar $T_{gel}$, which is close to the corresponding VPTT, revealing that the driving force for the sol-to-gel transition of the microgel dispersions should be mainly the hydrophobic interactions among the shrunk microgels, which only emerge after the temperature reaches close to the VPTT. Similar to the negligible effect of electrostatic interactions on the phase transitions of the persulfateinitiated polyNIPAM microgel (the KPS/NIPAM molar ratio $\approx$ 0.017) dispersions\(^{12}\), we speculate that the electrostatic interaction should be also negligible in the gelation process of our poly(NIPAM-co-AAm) microgel dispersions, since the small amount of sulfate groups derived from APS in the synthesis have little effect on the stability of the microgels as discussed above. This hypothesis is clearly
demonstrated by the perfect match of the dynamic stress sweep curves of the microgel dispersions at a very low (0.005 M) and a high (0.154 M) ionic strength (Figure 3–5).

Figure 3–6 Temperature dependence of dynamic modulus ($G'$: ■; $G''$: ●) of 4 wt% poly(NIPAM-co-AAm) microgel dispersions (pH = 7.4, and ion strength = 0.154 M): (A) NM1, (B) NM2, (C) NM3, (D) NM4, and (E) NM5. All measurements were made at a stress of 0.05 Pa and an oscillation frequency of 0.1 Hz.
To further understand the sol-to-gel phase transitions of the microgel dispersions, we determine the critical concentration of poly(NIPAM-co-AAm) microgel particles, above which the microgel dispersions are capable of forming a solid-like supra-structure at 37.0 °C. For hard-sphere dispersions, the particle concentration is quantified by the volume fraction $\phi$. However, because the microgel particles are deformable and their volume is not always a constant, $\phi$ is no longer a good measure of concentration. Instead, we use an effective volume fraction $\phi_{\text{effective}}$, which is always proportional to the polymer concentration, to describe the concentration of microgel particles. As shown in Figure 3–7, the microgels NM1, NM2, NM3, NM4, and NM5 of increased particle size displayed an increased critical $\phi_{\text{effective}}$. Generally, the pair potential between neutral or quasi-neutral microgel particles includes a short-range repulsion that is similar to the interaction between two polymer-coated surfaces, and a longer-ranged van der Waals-like attraction that arises from the difference in the Hamaker constants of the microgel particle and the solvent. Because the phase diagram is not sensitive to the detailed function of the short-range forces, Hu’s group proposed that the potential between microgel particles can be effectively represented by the Sutherland-like function:

$$
\frac{u(r)}{kT} = \begin{cases} 
\infty & r < \sigma \\
-\frac{T_0}{T} \left( \frac{\sigma_0}{\sigma} \right)^{m} \left( \frac{\sigma}{r} \right)^{m} & r \geq \sigma 
\end{cases}
$$

(3–2)

where $\sigma$ is the effective diameter, $r/\sigma$ denotes the reduced center-to-center distance, $k$ is the Boltzmann constant, $T$ is the absolute temperature, $T_0$ is an empirical proportionality constant that has the unit of temperature, and $\sigma_0$ is the particle diameter at a reference temperature where the conformation of the network chains is closest to that of unperturbed Gaussian chains. The $m$ value can be assumed to be 8, in considering that the van der Waals attraction between microgel particles is short ranged in comparison to that between atomic molecules (relative to the particle
Thus, for the monodispersed poly(NIPAM-co-AAm) microgels of a stable radius \( R_h \) at a fixed temperature of 37.0 °C, the function below for \( r \geq \sigma \) can be simplified as

\[
 u(r) = -\frac{kT_\sigma \sigma^{14}}{\sigma^{6}} \left( \frac{1}{r} \right)^{6} = -\frac{K^*}{\sigma^6} \left( \frac{1}{r} \right)^{6}
\]

where \( K^* \) is a constant including all the physical parameters mentioned above. \( K^* \) is always positive for two identical particles\(^{44}\), hence the interaction \( u(r) \) is always attractive. At the same center-to-center distance \( r \) between microgel particles, the interaction \( u(r) \) decreases inversely as the \( \sigma^6 \). Therefore, it is reasonable that the motion of poly(NIPAM-co-AAm) microgels of a smaller particle size is constrained at a relatively higher degree, resulting in the sol-to-gel transition appearing at a relatively lower particle concentration. Further support for our observation comes from an investigation by Segre’s group on the gelation of poly(methyl methacrylate) spheres, which were stearically stabilized by thin layers of poly(12-hydroxystearic acid)\(^{45}\). Thus, it is confirmed both experimentally and theoretically that strong attraction yields particles/clusters loosely packed. The stronger the interaction among the particles is, the lower the critical concentration for sol-to-gel transition of the particle dispersion. These results can not only provide important insights into the role that the size of microgel particles plays in controlling the phase transition, but also foreshadow a novel class of colloidal supra-structures for future applications.
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Figure 3–7. Observed phases of poly(NIPAM-co-AAm) microgel dispersions (pH = 7.4, and ion strength = 0.154 M) at 37.0 °C, showing the effect of particle size on the critical $\phi_{\text{effective}}$ for sol-to-gel transition.

3.3.4 Syneresis of the colloidal supra-structure

As-constructed colloidal supra-structures from the poly(NIPAM-co-AAm) microgel dispersions would shrink slightly accompanied with the expulsion of water from the networks, when brought above the corresponding VPTT of the microgel particles. As shown in Figure 3–8A, the colloidal supra-structure built from the smallest microgels of NM1 exhibited the largest degree of syneresis, at which the $(L_0-L_t)/L_0$ value can reach as high as 0.26 within our experimental time window. This result is in line with the most loosely packed structure due to the highest interparticle interactions as described by Eq. (3–3). In general, the colloidal supra-structures constructed from the microgels with an increased particle size exhibited a decreased degree of syneresis. Nevertheless, this syneresis phenomenon is quite similar to that found on polyNIPAM or polyAAm hydrogels, where the
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gel network is constructed by using polymer chains\textsuperscript{31-34}.

Figure 3–8 (A) Syneresis kinetics of the colloidal supra-structures as-constructed from 4 wt\% poly(NIPAM-co-AAm) microgel dispersions of pH 7.4 and ion strength 0.154 M: NM1 (■), NM2 (●), NM3 (▲), NM4 (▼), and NM5 (♦). All measurements were made at 37.0 °C. (B) Time dependence of $\Delta L_t/\Delta L_0$ during Syneresis. The solid lines are based on the fitting of Tanaka-Fillmore’s model as described in Eq. (3–4).
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The syneresis phenomenon of the colloidal supra-structure should be attributed to a combination effect of the deswelling behaviors from both the chemical network inside the microgel particles and the physical network of the connected microgel particles. Tanaka’s group found that the rate of volume change of a gel is scaled as \( l^{-2} \), where \( l \) is the characteristic length of a gel\(^{46}\). Thus, small gel particles should undergo a very fast volume phase transition. Indeed, nanosecond to microsecond (~100 ns) volume changes have been determined for individual temperature-sensitive polyNIPAM microgel particles in the size range of ~200-350 nm\(^4\). Considering the rapid volume phase transition of the individual microgel particles, we speculate that the kinetics of syneresis of the colloidal supra-structure should predominantly lie on the deswelling of the physical network of the connected poly(NIPAM-co-AAm) microgel particles. Figure 3–8B shows that the syneresis kinetic profiles of the colloidal supra-structures fit well to Tanaka-Fillmore’s model, which is well established to describe the swelling/deswelling kinetics of hydrogels\(^{46}\):

\[
\frac{\Delta L_t}{\Delta L_\infty} = \left(\frac{L_t - L_\infty}{L_0 - L_\infty}\right) = \left(\frac{L_t/L_0 - L_\infty/L_0}{L_0/L_\infty - L_\infty/L_0}\right) \propto \frac{1}{t^\tau}
\]

(3–4)

where \( L_\infty \) is the gel length at infinite time, and \( \tau \) is the characteristic time of swelling. The fitting curves exhibit a quick decrease in the time region of \( 0 < t < \sim 75 \) min and then reach almost a plateau when time \( t > \sim 75 \) min. From the fitting lines, the characteristic time \( \tau \) was estimated to be 23.01 min, 23.29 min, 23.57 min, 24.16 min, and 25.15 min, respectively, for the colloidal supra-structures built from the poly(NIPAM-co-AAm) microgels of NM1, NM2, NM3, NM4, and NM5 with a subsequently increased size. These results provide an further, though indirect, experimental proof that the designed physical networks of microgel particles can behave like the 3D networks of polymer chains. It is anticipated that the constructed
colloidal supra-structures with tunable syneresis properties should have great potential for many applications.

3.3.5 Cytocompatibility of the colloidal supra-structures

**Figure 3–9** Live/Dead assay of the colloidal supra-structures constructed from the poly(NIPAM-co-AAm) microgels of (A) NM1, (B) NM2, (C) NM3, (D) NM4, and (E) NM5, respectively, after 1, 4, 7, 14, and 21 days’ of cell seeding.

Having demonstrated the packing of poly(NIPAM-co-AAm) microgel particles to form
colloidal supra-structures of 3D crosslinked polymer-like nature, we carried out additional and independent tests on the designed colloidal supra-structures for cell scaffold application. B16F10 cells were selected as a model to evaluate the response of cells to the microgel-assembled scaffolds. As seen in Figure 3–9, the colloidal supra-structures provided structural support for the attachment of B16F10 cells and most of the cells were viable after 21 days of culture, although it was noticed that the colloidal supra-structures started to disintegrate when cell culture was prolonged beyond this time regime. The seeded cells migrated and homogeneously distributed throughout the physical networks of microgel particles. A similar behavior was observed in the cases of the 3D networks of polymer chains. Culture within a 3D gel environment can prevent dedifferentiation of the cells by maintaining these cells in a spindle-like morphology, as opposed to the spherical morphology found in two-dimensional (2D) monolayer culture. Viable cells increase continuously from day-4 to day-21, suggesting that the colloidal supra-structures can act as scaffolds to support cell proliferation.

An important feature is that the cell proliferation rate is highly depended on the colloidal supra-structure engaged. Typically, the colloidal supra-structures constructed from microgels of increased particle size displayed an increased cell proliferation rate. For example, after 7 days’ culture, it can be easily observed by naked eyes that cells cultured in the colloidal supra-structures built from larger microgels exhibited a much higher packing density (Figure 3–9). This result was further supported by MTT assay (Figure 3–10). To quantify the cell proliferation rate, the cell viability of the control group without any microgels at day-1 was assigned a value of 1, and the relative cell viability was determined relative to that. While a time dependent increase in relative cell viability was observed in all
cases, a greater enhancement in the relative cell viability was detected in the colloidal supra-
structures built from the larger microgel particles. We noted that the syneresis degree of the
colloidal supra-structures reveals the opposite trend towards the change in the size of the
building microgel particles (Figure 3–8). It is possible that a reduced syneresis degree is
favourable to the proliferation of cells, since it should be easier to transport oxygen and
nutrient, as well as metabolic waste, inside the networks. The limited supply in nutrient and
oxygen through the 3D networks may makes the cell proliferation become slower in 3D
scaffolds than that in 2D cultures, where cells are directly exposed to the nutrient- and
oxygen-rich media. This result is in accordance with the observation on hydrogels of
crosslinked polymer chains, which demonstrated that an increased permeability can enhance
cell proliferation\textsuperscript{15-17}. Different polymers have been explored to form hydrogels with
improved porosity and stiffness\textsuperscript{15-17}. Herein, we demonstrate that the size of building
microgel particles in the colloidal supra-structure can have important implications for
cellular behavior. Cultured in the colloidal supra-structures constructed from the microgels
NM1 or NM2 of small particle size (3D cultures), the proliferation rate of cells was slower
than that of the control group (2D culture). An gradual increase in the size of building
microgel particles (e.g., NM3, NM4, and NM5) could significantly enhance the cell
proliferation (Figure 3–10). This result imply that the permeability of the colloidal supra-
structure could be simply modulated through the size control of microgel building blocks to
alter the growth and survival characteristics of the cells \textit{in vitro}. 

Results of MTT assay, showing the cell viability with the colloidal supra-structures acting as cell scaffolds, which were constructed from 4 wt% of poly(NIPAM-co-AAm) microgels NM1 (■), NM2 (●), NM3 (▲), NM4 (▼), and NM5 (♦), respectively. The result of a control group without adding any microgels (□) is also presented for comparison.

3.4 Conclusion

We have demonstrated that the colloidal supra-structures could be constructed by the thermo-driven gelation of the colloidal dispersion of poly(NIPAM-co-AAm) microgels. Different from the previous examples in which the VPTT of the temperature-responsive microgels are much below 37 °C, making them fully collapsed and possibly hard-sphere-like under the physiological conditions, the presented poly(NIPAM-co-AAm) microgels exhibit a reversible and continuous volume transition in water with VPTT ≈ 35 °C and remain partially swollen and soft under physiological conditions. The constructed colloidal supra-structure can be regarded as a model system for a new class of cell scaffolds. More importantly, we
found that the size of the microgel particles can affect the sol-to-gel phase transition of the microgel dispersions, alter the syneresis degree of the constructed colloidal supra-structures, and tailor the cytocompatibility when the colloidal supra-structures were used for cell scaffolds. The relationship between the size of the building microgel particles and the cytocompatibility of the constructed colloidal supra-structure could provide fundamental guidance for the construction of desirable scaffolds to regulate the cell proliferation rate. We anticipate that the aforementioned approach may serve as a good starting point for the design of functional biomaterials with supramolecular chemistry.
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3.5 References


Chapter 4

Tailored Core-Shell Microgels for Highly Efficient Curcumin Delivery Carrier

4.1 Introduction

Curcumin, a yellow compound presented in spice turmeric (Curcuma longa), has drawn considerable attention of research in recent years by showing safety and diverse pharmacologic effects\textsuperscript{1-3}. Curcumin can prevent carcinogen-induced tumorigenesis and inhibit growth of many tumor cell lines\textsuperscript{4-7}. Unfortunately, clinical advancement of this promising compound has been hindered by its exceedingly low water solubility, rapid metabolism in the body, and low oral bioavailability\textsuperscript{8-9}. To bring this safe and affordable natural product to the forefront in the fight against cancers and other chronic diseases, it is critical to develop efficient delivery platforms that can carry sufficient curcumin molecules and attain pharmacological effect.

A variety of delivery vehicles have been recently pursued to enhance the curcumin delivery, including liposomes\textsuperscript{10-17}, micelles\textsuperscript{18-22}, phospholipid or cyclodextrin complexes\textsuperscript{23-27}, polyelectrolyte microcapsules\textsuperscript{28}, and polymer formulated nanoparticles (NPs)\textsuperscript{29-47}. Studies on these delivery vehicles have provided a promising direction to improve the total content of
curcumin in aqueous media, increase the curcumin stability, and enhance the cellular uptake. However, the development of NP systems for curcumin delivery is still in its infancy. First, the loading capacity of the currently developed nanocarriers for curcumin is still very low. Second, the nanocarriers based on the liposomes, micelles, and complexes from the association of surfactants, lipids, amphiphilic polymers or polysaccharides tend to be metastable. They may disassemble quickly in the body, thus limit the circulation time of curcumin carriers in vivo. Third, the currently developed nanocarriers are generally insensitive to the environmental condition change, thus cannot intelligently trigger the release of curcumin. A key attribute of drug delivery systems is their ability to regulate the drug release to improve therapeutic efficacy.

The use of microgels as drug delivery vehicle has opened new avenues for the biological and biomedical applications. As a drug delivery carrier, polymer microgels is one of the best candidates because of their tunable size from nanometers to micrometers, favorable biocompatibility, a large surface area for multivalent bioconjugation with targeting ligands, and an interior network for the incorporation and protection of therapeutics. Furthermore, responsive polymer microgels can undergo stimuli-responsive volume transitions, which enable the fabrication of “smart” carrier systems that can release a drug in response to environmental stimuli, such as a change in pH, temperature, light and electrical field. Microgels loaded with drugs by encapsulation, surface attachment or entrapment can efficiently penetrate into individual cells because of their small sizes. If stimuli applied, the microgels will change the swelling degree and release the drugs out, resulting in efficient drug accumulation at the target site. This process provides a prolonged release of the drug, minimizes unwanted side effects, thus improving the therapeutic efficacy of conventional pharmaceuticals. In addition, rationally designed microgels with special compositions and nanostructures can deliver some
poor soluble or unstable therapeutic agents, which can sufficiently increase the aqueous solubility of drug, protect the drug from degradation, and thus improve the bioavailability of the drug\textsuperscript{56}.

In this work, we report a novel type of core-shell structured microgels that exhibit monodispersed spherical bead morphology and tunable temperature sensitive volume phase transitions for highly efficient curcumin delivery carriers with high drug loading yields and fluorescence imaging ability of cancer cells. As shown in Figure 4–1, the core-shell microgels are composed of the poly(2-vinylanisole) (PVAS) nanogel as a core and the nonlinear poly(ethylene glycol) (PEG) copolymer gel as a hydrophilic shell. Considering the unique molecular structure of curcumin, we have selected the hydrophobic PVAS gel as a core so that the curcumin molecules can interact with the core nanogel effectively through the $\pi-\pi$ stacking and other hydrophobic interactions due to the structural similarity, which is expected to enhance the drug loading capacity significantly. On the other hand, the nonlinear PEG polymers containing short oligo(ethylene glycol) side chains have been found to exhibit an interesting phase transition behavior in aqueous solution with a tunable lower critical solution temperature (LCST) through the control in the compositions of the oligo(ethylene glycol) side chains\textsuperscript{59–62}. The longer the oligo(ethylene glycol) side chain, the higher the LCST of the nonlinear PEG polymers. Because of the thermo-sensitive, water-soluble and biocompatible properties\textsuperscript{60}, these nonlinear PEG-based polymers are regarded as a very promising material for the construction of polymeric drug carriers. In order to enable the core-shell microgel drug carriers to be responsive in the physiologically important temperature range, we have selected the 2-(2-methoxyethoxy)ethyl methacrylate (MEO\textsubscript{2}MA) and oligo(ethylene glycol) methyl ether methacrylate ($M_n = 300\ \text{g/mol}$, MEO\textsubscript{5}MA) as comonomers in a ratio of 1:2 to synthesize the
nonlinear PEG gel shell. The shell thickness can be well controlled through the core-shell feeding ratios in synthesis. We expect that the resulted PVAS@PEG core-shell structured microgels will exhibit excellent water dispersibility in the physiologically important temperature range or below, which allows the administration of curcumin in an aqueous medium. More importantly, the variation of temperature can control the release rate of curcumin from the microgel particles. The effects of shell thickness on the drug loading capacity, sustained drug release behavior, and cytotoxicity of the drug-free and drug-load microgels were evaluated. The results presented in this work shows that such rationally designed water dispersible PVAS@PEG core-shell microgels provide high curcumin loading capacity, triggerable sustained release of curcumin, good cell penetration ability, and high therapeutic efficacy as the drug delivery carrier.

Figure 4–1 Typical Schematic illustration of curcumin-loaded core-shell microgels with PVAS gel as a core and the nonlinear PEG gel as a shell. The hydrophobic curcumin drug is encapsulated within the inner PVAS core, which is coated by an outer PEG-based shell to offer both stability in aqueous media and temperature sensitivity. Such designed core-shell microgels-based drug delivery system has the potential to render the hydrophobic curcumin dispersible in aqueous media, thus overcome the barriers of poor solubility of curcumin in water.
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4.2 Experimental

4.2.1 Materials

All chemicals were purchased from Aldrich. 2-(2-methoxyethoxy)ethyl methacrylate (MEO₂MA, 95%), oligo(ethylene glycol)methyl ether methacrylate (MEO₃MA, \( M_n = 300 \text{ g/mol} \)) and poly(ethylene glycol) dimethacrylate (PEGDMA, \( M_n \approx 550 \text{ g/mol} \)) were purified with neutral Al₂O₃. Curcumin was purified with anhydrous ethanol. Anhydrous ethanol, 2-vinylanisole, divinylbenzene (DVB), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium dodecyl sulfate (SDS), Dulbecco's Modified Eagle Medium (DMEM), and Fetal bovine serum (FBS) were used as received without further purification. The water used in all experiments was of Millipore Milli-Q grade.

4.2.2 Synthesis of poly(2-vinylanisole) core microgels

The core microgels were prepared by free radical precipitation copolymerization of 2-vinylanisole using AAPH as an initiator. A mixture of 2-vinylanisole \((5.96 \times 10^{-4} \text{ mol})\), DVB \((5.61 \times 10^{-5} \text{ mol})\), SDS \((3.46 \times 10^{-4} \text{ mol})\), and water \((95 \text{ mL})\) was poured into a 250 mL three-neck round-bottom flask equipped with a stirrer, a nitrogen gas inlet, and a condenser. After 30 min, the temperature was raised to 70 °C and the polymerization was initiated by adding 1 mL of AAPH \((0.105 \text{ M})\). The polymerization was allowed to proceed for 5 h. The resulted solution was centrifuged three times at 10,000 rpm (30 min, Thermo Electron Co. SORVALL® RC-6 PLUS superspeed centrifuge) with supernatant discarded and the precipitate redispersed in 200 mL deionized water. The resultant poly(2-vinylanisole) (abbreviated to PVAS) microgels were used as nuclei for subsequent precipitation polymerization to add the nonlinear PEG copolymer gel shell of P(MEO₂MA-co-MEO₃MA).
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4.2.3 Synthesis of PVAS@PEG core-shell microgels

The outer shell precursors of MEO$_2$MA and MEO$_5$MA comonomers mixture in 1:2 molar ratio and PEGDMA crosslinker were dissolved into the 100 mL purified PVAS nanogel dispersion. The mixture was heated to 70 °C under a N$_2$ purge. After 30 min, 1 mL of AAPH (0.105 M) initiator was added to start the polymerization. The synthesis was allowed to proceed to total 5 h. The resulted PVAS@PEG core-shell microgels were purified with centrifugation/redispersion in water for three cycles, followed by 3 days of dialysis (Spectra/Pro® molecularporous membrane tubing, cutoff 12,000–14,000) against very frequently changed water at room temperature (~22 °C). Different feeding compositions for the synthesis of the PVAS@PEG core-shell microgels are listed in Table 4–1 to control the different shell thickness. The PVAS@PEG core-shell microgels are coded as VEM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Core Solution (mmol)</th>
<th>Shell Solution (mmol)</th>
<th>$R_h$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA core microgel</td>
<td>0.594</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>PEG-based shell microgel</td>
<td></td>
<td>0.75</td>
<td>2.26×10$^{-2}$</td>
</tr>
<tr>
<td>VEM1</td>
<td>0.594</td>
<td>0.20</td>
<td>0.60×10$^{-2}$</td>
</tr>
<tr>
<td>VEM2</td>
<td>0.594</td>
<td>0.25</td>
<td>0.75×10$^{-2}$</td>
</tr>
<tr>
<td>VEM3</td>
<td>0.594</td>
<td>0.50</td>
<td>1.51×10$^{-2}$</td>
</tr>
<tr>
<td>VEM4</td>
<td>0.594</td>
<td>0.75</td>
<td>2.26×10$^{-2}$</td>
</tr>
</tbody>
</table>

$R_h$ measured by Dynamic light scattering (DLS) method at 22 °C and a scattering angle of $\theta = 45^\circ$.
4.2.4 Drug loading and release

Curcumin was loaded into the microgels mainly driven by hydrophobic interactions. 5 mL microgel dispersion was stirred in an ice water bath for 30 min. 4 mL fresh curcumin solution of 1mg/mL in anhydrous ethanol was then added dropwisely to the vial. After stirring overnight, the suspension was centrifuged at 10,000 rpm for 15 min at 22°C. To remove free curcumin, the precipitate was redispersed in 5 mL water, and further purified by repeated centrifugation and washing for at least six times. All the upper clear solutions were collected, and the concentration of free curcumin was determined by UV-vis spectrometry at 435 nm. The emission intensity at 566 nm with the excitation wavelength of 420 nm on the upper clear solutions was also recorded to confirm the loading amount. Optical signal was converted to concentration based on the linear calibration curve $R^2 > 0.99$ measured using the curcumin solution with known concentrations under the same condition. The amount of loaded curcumin in the microgels was calculated by deducting curcumin amount in upper clear solution from the total curcumin amount (4 mg). The loading capacity is expressed as the mass of loaded drug per unit weight of dried microgels. The loading experiments at physiological temperature 37°C were also performed by using the same procedure as that at 22°C.

The in vitro release test of curcumin from the microgels was evaluated by the dialysis method. The curcumin-loaded microgel dispersion was diluted to 0.15 mg/mL for the release experiments. A dialysis bag (Spectra/Pro® molecularporous membrane tubing, cutoff 12,000–14,000) filled with 1 mL diluted curcumin-loaded microgels was immersed in 50 mL 0.005 M phosphate buffer solutions (PBS) of pH = 6.15 at different temperatures. The released curcumin outside of the dialysis bag was sampled at defined time period and assayed by
fluorescence spectrophotometer at 566 nm upon excitation at 420 nm. Cumulative release is expressed as the total percentage of drug released through the dialysis membrane over time.

4.2.5 Incorporation of microgels into mouse melanoma cells B16F10

The microgels pre-loaded with curcumin undergo a releasing experiment at 37 °C for 7 days until no curcumin would further release out from the microgels, in which about 20% of curcumin was still entrapped to provide fluorescence signal for cellular imaging.

Round glass coverslips were placed in wells of a 24-well plate and treated with 0.1% poly-L-lysine in 100 mM PBS for 40 min. Following the treatment, the solution was aspirated and the wells were washed with PBS 3 times each. Next, B16F10 cells were plated on the glass coverslips at 80% confluence in DMEM containing 10% FBS and 1% penicillin-streptomycin. After 24 h, 500 µL of different curcumin-loaded microgels (0.3 µg/mL) in serum-free DMEM were added into the wells respectively. The plate was incubated at 37 °C for 2 h. The medium was then aspirated and fresh serum-free DMEM was added to each well. Finally, the coverslips with cells were removed from the wells and mounted onto slides for confocal microscopy study.

4.2.6 In vitro cytotoxicity

B16F10 cells (6 × 10³ cell/well) were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin in a 96-well plate, and exposed to free curcumin, curcumin-free microgels, and curcumin-loaded microgels, respectively. The plate was washed three times using fresh serum-free DMEM. The plate was incubated at 37 °C for 2 h. After that, 25 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in phosphate buffered saline (PBS)) were added to the wells. After incubation for 2 h, the solution was
aspirated and 100 µL of DMSO was added to each well to dissolve the formazan crystal, and the plate was sealed and incubated overnight at 37 °C with gentle mixing. Three portions of the solution obtained from each well were transferred to three respective wells of a 96-well plate. Cell viability was measured using a microplate reader at 570 nm. Positive controls contained no drug or microgels, and negative controls contained MTT.

4.2.7 Characterization

The morphology of the core-shell microgels was characterized with transmission electron microscopy (TEM). The TEM images were taken on a FEI TECNAI transmission electron microscope at an accelerating voltage of 120 kV. Approximately 10 µL of diluted microgel suspension was dropped on a Formvar-covered copper grid (300 meshes) and then air-dried at room temperature for the TEM measurements. The UV-vis absorption spectra were obtained on a Thermo Electron Co. Helios β UV-vis Spectrometer. The PL spectra were respectively obtained on a JOBIN YVON Co. FluoroMax®-3 Spectrofluorometer equipped with a Hamamatsu R928P photomultiplier tube, calibrated photodiode for excitation reference correction from 200 to 980 nm, and an integration time of 1 s. The pH values were obtained on a METTLER TOLEDO SevenEasy pH meter. The B16F10 cells incorporated with microgels were imaged using a confocal laser scanning microscopy (LEICA TCS SP2 AOBS™) equipped with an HC PL APO CS 20 × 0.7 DRY len. A UV (405 nm) light was used as the light source.

Dynamic light scattering (DLS) was performed on a standard laser light scattering spectrometer (BI-200SM) equipped with a BI-9000 AT digital time correlator (Brookhaven Instruments, Inc.). A Nd:YAG laser (150 mW, 532 nm) was used as the light source. All microgel solutions were passed through Millipore Millex-HV filter with a pore size of 0.80 µm.
to remove dust before the DLS measurements. In DLS, the Laplace inversion of each measured intensity-intensity time correlated function can result in a characteristic line width distribution $G(\Gamma)$\textsuperscript{63}. For a purely dissuasive relaxation, $\Gamma$ is related to the translational diffusion coefficient $D$ by $(\Gamma/q^2)_{\rightarrow 0,q^2 ightarrow 0} = D$, where $q = (4\pi n/\lambda)\sin(\theta/2)$ with $n$, $\lambda$, and $\theta$ being the solvent refractive index, the wavelength of the incident light \textit{in vacuo}, and the scattering angle, respectively. $G(\Gamma)$ can be further converted to a hydrodynamic radius ($R_h$) distribution by using the Stokes-Einstein equation, \[R_h = (k_B T/6\pi \eta)D^{-1},\] where $k_B$, $T$, and $\eta$ are the Boltzmann constant, the absolute temperature, and the solvent viscosity, respectively.

4.3 Results and discussion

4.3.1 Synthesis and structure of PVAS@PEG core-shell microgels

Our strategy to prepare the PVAS@PEG core-shell microgels involves the first synthesis of a PVAS core microgel, followed by the synthesis of the nonlinear PEG gel layer on the core microgel. The PVAS core microgels were synthesized based on the well-established precipitation polymerization method. Because of low solubility of 2-vinylanisole in water, the PVAS fragments formed at the early stage of reaction would nucleate and precipitate into the hydrophobic alkyl chain regions of the SDS molecules. With the proceeding of polymerization reaction, more PVAS fragments were added onto the initially formed hydrophobic gel layer, leading to a continuous growth in size until the polymerization reaction was completed. The as-synthesized hydrophobic PVAS core microgels with $R_h = 70$ nm at 22 °C were used as a seed for further polymerization to form the outer shell layer of nonlinear PEG-based gel, which consists of an oligo-PEG crosslinked copolymer of MEO$_2$MA and MEO$_5$MA.
Figure 4–2 shows the size distributions of the PVAS@PEG core-shell microgels, in terms of the hydrodynamic radius ($R_h$) measured at $T = 22 \, ^\circ C$ and $\theta = 45^\circ$, synthesized with different feeding amount of MEO$_2$MA and MEO$_5$MA but at the same MEO$_2$MA : MEO$_5$MA molar ratio (1:2). All the obtained PVAS@PEG core-shell microgels have a very narrow size distribution. The DLS characterization indicates that the obtained microgels are nearly monodispersed with a polydispersity index of $\mu^2/\langle \Gamma^2 \rangle = 0.001$. As can be seen in Table 4–1, the hydrodynamic radius ($R_h$) of the core-shell microgels VEM1, VEM2, VEM3, and VEM4 synthesized using the same core microgel template are 101, 110, 138, and 165 nm, respectively, indicating that the microgel particle size increases with the increase in the feeding amount of shell precursors of MEO$_2$MA and MEO$_5$MA. This result indicates that the shell thickness and size of the core-shell microgel can be controlled by a simple change of the feeding amount of comonomers for shell synthesis as the application needs.
Figure 4–3 shows the typical TEM images of the dried core-shell microgels VEM2 and VEM3 (see Table 4–1). Both microgels exhibit monodispersed spherical bead morphology and a well-defined core-shell architecture with a clear boundary between the core and shell. The core-shell structure can be clearly observed with a dark condensed core and a light contrast shell. The dark core can be attributed to the higher electron density of the PVAS chains and the shorter crosslinker DVB (equivalent to higher crosslinker density) than the oligo-PEG crosslinked nonlinear PEG gel shell. The clear core-shell structure is associated with the fact that the hydrophobic densely crosslinked PVAS core can hinder the relatively hydrophilic nonlinear PEG-based chains of the outer shell from interpenetrating into the inner core area. The TEM images also clearly demonstrate that the shell of VEM2 microgel is much thinner than that of the
VEM3 microgel, further proving that the shell thickness of the core-shell microgels can be tuned by simply adjusting the feeding ratio of the shell precursors to PVAS core in the synthesis.

4.3.2 Temperature-responsive volume phase transitions of the core-shell microgels

![Graph showing temperature dependence of the average $R_h$ values of PVAA@PEG core-shell microgels VEM1 (■), VEM2 (●), VEM3 (▲), and VEM4 (▼), measured at a scattering angle $\theta$ = 45°.]

Figure 4-4 shows the temperature-induced volume phase transitions of the PVAS@PEG core-shell microgels in terms of the change of $R_h$ measured at a scattering angle of $\theta$ = 45°. It is clear that the increase in temperature of dispersion medium can induce a significant shrinkage of the core-shell microgels. The hydrophobic PVAS core would not undergo conformational and chemical changes in water when giving external stimuli of temperature change. The observed temperature-induced volume phase transitions of the core-shell microgels should be attributed to
the nonlinear PEG gel shell containing P(MEO₂MA-co-MEO₅MA) chains. It is believed that the water solubility of this type of nonlinear PEG-based copolymer comes from the formation of hydrogen-bonds between ether oxygen of the side PEG chains and the hydrogen of water. On the other hand, the hydrophobicity of the apolar backbone would counterbalance this favorable effect in water⁵⁹,⁶⁴. This counterbalance of the hydrophilic and hydrophobic forces results in the swelling/deswelling characteristic of our microgels. The critical volume phase transition temperature (VPTT) of the core-shell microgels can be controlled by the feeding molar ratio \( r_{\text{mol}} = n_{\text{MEO2MA}}/n_{\text{MEO5MA}} \) in shell gel synthesis. The lower the \( r_{\text{mol}} \), the higher the VPTT of the microgels will be⁶⁴. Figure 4–4 indicates that the proper feeding molar ratio of 1:2 between the two macromonomers of MEO₂MA and MEO₅MA has led to a continuous change in hydrodynamic radius (\( R_h \)) with tunable slopes across the physiologically important temperature range of 37–42 °C, which are typical abnormal temperature range found in many pathological zones such as tumors⁶⁵–⁶⁷. Additionally, the microgels did not reach a fully collapsed state at the experimental temperature limit up to 50 °C, thus the partially swollen PEG-based outer gel layer is still hydrophilic at temperature below 50 °C. This hydrophilic gel shell makes the PVAS@PEG core-shell microgels very stable in water. Even after few months, no sediment was observed in the microgel solutions, which is very important to serve as a drug carrier for delivery of hydrophobic drugs. While the inner PVAS core provides hydrophobic region for the storage of hydrophobic drugs, the hydrophilic outer PEG-based gel shell can enable the microgels to disperse in cell culture medium and further penetrate into the cells. The thermo-responsive outer PEG-based gel shell can be utilized to regulate the transport of the drug molecules from the inner gel core to the surrounding medium by temperature stimuli.
4.3.3 Drug loading capacity of the PVAS@PEG core-shell microgels

The hydrophobic PVAS core of the core-shell microgels offers the capability for storage of hydrophobic drugs. We expect that the PVAS core with similar structural units to the curcumin molecules should have strong hydrophobic interactions to attract the curcumin molecules into the core region. Curcumin is poorly soluble in water at acidic or neutral pH with the macroscopic undissolved flakes visible in the solution\(^1\), while at pH above neutral value it would undergo rapid hydrolytic degradation\(^8\),\(^68\),\(^69\) and thus lose the pharmaceutical effects. It has been determined that the half-time for the hydrolytic degradation of curcumin in aqueous solution containing 10% organic solvent at pH \(\approx 6\), 7, and 8 is \(4.2 \times 10^3\) h, 15 h and \(3.5 \times 10^{-2}\) h, respectively\(^69\). Therefore, we loaded the curcumin molecules into the PVAS@PEG core-shell microgels dispersed in distilled water at pH = 5.7, where the curcumin molecules should have no significant degradation occurred.

![Graph showing curcumin loading capacity as a function of shell thickness.](image)

**Figure 4–5** Curcumin loading capacity of the PVAS@PEG core-shell microgels as a function of shell thickness, measured at 22 °C.
Figure 4–5 shows the effect of shell thickness on the drug loading capacity of the core-shell microgels measured at 22 °C. For PVAS core, VEM1, VEM2, VEM3, and VEM4 core-shell microgels, the loading capacity was determined as 18.5 wt%, 29.3 wt%, 31.9 wt%, 35.5 wt%, and 38.0 wt%, respectively. The thicker the outer PEG-based gel shell, the higher the loading capacity of the core-shell microgels. When the shell thickness is above 40 nm, the loading capacity still increases with the increase in shell thickness, but with a slower pace. If we further increase the shell thickness, the loading capacity would increase at a much slower pace. This result indicates that very little curcumin molecules were associated with PEG-based gel shell. Indeed, this was confirmed by the extremely low loading capacity of pure PEG-based microgels (see Table 4–1) of 1.52 wt%. In contrast, the pure PVAS core demonstrates a much higher curcumin loading capacity (18.5 wt%) than the pure nonlinear PEG-based microgels with the same composition as the gel shell. Thus, the drug loading is mainly contributed by the PVAS core, whose hydrophobic aromatic groups can form strong hydrophobic associations with the aromatic phenols in curcumin molecules to form intermolecular complexes, resulting in relative high drug loading capacity. Nevertheless, it should be noticed that the curcumin loading capacity of the pure PVAS core microgels is much lower than that of the PVAS@PEG core-shell structured microgels. The high curcumin loading capacity of the core-shell structured microgels implies that there must be another important factor to enhance the drug loading capacity, which should be the collapsing degree (mesh size of network) of the PVAS core microgels. The PVAS core microgels are hydrophobic and densely shrunk together. Such a collapsed structure has limited space to hold the curcumin drug molecules. However, in the core-shell nanostructured microgels, the introduction of the hydrophilic and swollen gel shell layer can restrict the PVAS core microgel from collapsing. The swollen shell can pull up and stretch the core network to
make a bigger mesh size even though the core network chains are still hydrophobic. In this case, the open network with large mesh size of the hydrophobic PVAS core could hole much more hydrophobic curcumin molecules. The thicker the swollen nonlinear PEG gel shell, the larger the pulling force to restrict the collapsing of the hydrophobic PVAS core chain network, resulting in higher curcumin loading capacity of the core-shell microgels. When the shell thickness is increased to a certain value, the mesh size of the hydrophobic PVAS core network chains will no longer increase due to the limitation of chemical crosslinking. At that moment, the drug loading capacity of the core should reach to a maximum value. The further increase in the thickness of PEG gel shell would only increase the overall loading capacity of the core-shell microgels slightly because the PEG gel shell has very low loading capacity for hydrophobic curcumin drug.
4.3.4 *In vitro* release studies

![Graphs showing cumulative release over time at different temperatures for curcumin release from core-shell microgels VEM1 (A), VEM2 (B), VEM3 (C), and VEM4 (D).](image)

**Figure 4–6** Releasing profiles of curcumin from the core-shell microgels VEM1 (A), VEM2 (B), VEM3 (C), and VEM4 (D) at different temperatures. Curcumin has been pre-loaded into core-shell microgel at 22 °C. In the blank (◊), 1 mL diluted solution of free curcumin (containing 5% ethanol) with an equivalent amount of drug to the trapped in VEM1 was performed at 41 °C. All releasing experiments were carried out in 50 mL PBS (0.005M) of pH = 6.15.
Figure 4–6 shows the release kinetics of curcumin from the VEM1, VEM2, VEM3, and VEM4 core-shell microgels. The in vitro release test was carried out in a PBS of pH = 6.15 to avoid the evident degradation of curcumin from the long time exposure in water. A blank release experiment of free curcumin (D ≈ 379 Da) solution (containing ~5% ethanol) with an equivalent amount of drug to that trapped in VEM1 was also performed, showing that the dialysis membrane (cutoff 12000 – 14000 Da) played a negligible role in the release kinetics (Figure 4–6A). Three features should be noted. Firstly, the curcumin release from the core-shell microgels is much slower than from the free curcumin solution, indicating a sustained release of curcumin from the core-shell microgels. Secondly, the release kinetics of curcumin from the core-shell microgels was temperature dependent. The release of curcumin could be significantly speeded up by increasing temperature for all the VEM1, VEM2, VEM3 and VEM4 core-shell microgels. For instance, only 16.1% of curcumin was released from VEM1 at 22 °C for 72 h. When temperature of medium increased to 37, 39, and 41 °C, the percentage of curcumin released from VEM1 reached to 44.4%, 65.3%, and 80.4% during the same time period (72 h). This temperature dependence of curcumin release should be attributed to thermo-responsive nonlinear PEG gel shell. The increase in temperature induced a gradual shrinking of the PEG gel shell and thus reduced the pulling force of shell to restrict the collapsing of hydrophobic core, which compressed the core space and thus squashed out the curcumin molecules. Meanwhile, the shrunk PEG gel shell could also reduce the restricted diffusion path length of curcumin molecules from the core region to the medium outside the microgels. The higher the temperature of the dispersion medium, the thinner the PEG gel shell, which compressed more on the PVAS core, thus more curcumin molecules could be released out from the core-shell microgel. Thirdly, a slightly quicker release rate was determined at all the investigated temperatures for core-shell
microgels with thinner PEG-based shell. For example, the percentage of curcumin released from VEM1, VEM2, VEM3 and VEM4 at 41 °C for 72 h was 80.4%, 78.0%, 75.9%, and 73.7%, correspondingly. This result could be also attributed to the factor that the thin gel shell can shorten the restricted diffusion path of the curcumin from the core to external dispersion medium.

![Figure 4-7](image)

**Figure 4-7** Releasing profiles of curcumin from the core-shell microgels VEM2 (A) and VEM3 (B) at different temperatures. Curcumin has been pre-loaded into core-shell microgel at 37 °C. All releasing experiments were carried out in 50 mL PBS (0.005M) of pH = 6.15.

In practical applications, the drug carriers should have a minimum release of drug at normal body temperature of 37 °C. The core-shell microgels loaded with curcumin at 22°C demonstrate a significant release of curcumin at 37 °C. To further explore the drug releasing property of the core-shell microgels at physiologically important temperature range of 37–42 °C, we loaded the curcumin into the core-shell microgels at 37 °C and then did the *in vitro* releasing test at different temperature of 37, 39 and 41 °C, respectively. Figure 4–7 shows the release kinetics of curcumin from the VEM2 and VEM3 core-shell micogels loaded with curcumin at 37 °C.
While the general trend of temperature-triggerable drug release remains the same as observed in Figure 4–6, only 15.6% and 12.9% curcumin was respectively released from VEM2 and VEM3 at 37 °C, which is very important to minimize the drug release at normal condition. When the temperature of releasing medium was increased to physiologically abnormal temperature of 39 °C and 41 °C, the release of curcumin was significantly increased. Thus, the temperature-regulated drug release could be exploited for intelligent drug delivery applications through environmental stimuli. The abnormal temperature rise in pathological zones could facilitate active drug release from the core-shell microgels.

4. 3. 5 Hydrolytic Degradation of Curcumin

Studies have shown that the hydrolytic degradation process of curcumin occurs rapidly at a pH above neutral. Wang et al. have used high performance liquid chromatography (HPLC) and mass spectrometry to show that curcumin is partially deprotonated initially, which is followed by fragmentation into trans-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexanal as the main product, then further decomposed to vanillin, ferulic acid, and feruloyl methane. Mandy et al. have determined that the condensation products contribute negligible to the total absorption signal. Thus the degradation of curcumin can be determined from the decrease of visible absorbance over time, which signifies the decrease of curcumin concentration.
Figure 4–8 Fluorescence spectra of curcumin in pH = 7.4 PBS buffer (A) and curcumin-loaded VEM3 (B) over a course of 180 min. The decays of the curcumin at the absorption maxima are shown in C (●: curcumin-loaded VEM3, ■: pH = 7.4 PBS buffer). All experiments were carried out at 37 °C.

Degradation of curcumin was recorded over 180 min at 37 °C and 15 min intervals by monitoring the decrease in the absorption maximum and the results are shown in Figure 4–8. In pH = 7.4 PBS buffer solution (Figure 4–8A), the absorption maximum decays to approximately 40% of initial value in 180 min. In contrast, the rate of degradation in curcumin-loaded VEM3
are significantly lower (Figure 4–8B), where the absorption at maximum decreases to about 85% of initial value. Furthermore, we showed before that about 10% of curcumin had been released out from VEM3 after 3 h at 37 °C (Figure 4–6C), which might degrade in PBS solution. These results indicated the PVAS@PEG core-shell microgels can significantly restrict the degradation of curcumin and improve therapeutic efficacy.

4.3.6 Tumor cell imaging

![Scanning confocal fluorescence images of mouse melanoma B16F10 cells upon staining](image)

**Figure 4–9** Scanning confocal fluorescence images of mouse melanoma B16F10 cells upon staining with curcumin loaded core-shell microgels VEM1 (A), VEM2 (B), VEM3(C), and VEM4 (D), respectively. Excitation wavelength is 496 nm.
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Curcumin can emit strong visible fluorescence under the physiological conditions, which renders them useful for biomedical detection of the drug carriers and imaging diagnosis. We loaded curcumin to core-shell microgels and then release them at 37 °C for 7 days. This procedure ensured no free curcumin molecules would further release out from the core-shell microgels when we incorporate these microgels into the tumor cells, while the curcumin molecules still trapped in the core of microgels can emit fluorescence signals for cellular imaging to examine whether these core-shell microgel particles can permeate into the cells. Figure 4–9 shows the scanning confocal fluorescence images of the mouse melanoma cells B16F10 after stained with curcumin-loaded core-shell microgels for 2 h, obtained from the laser confocal microscopy. The bright spots are attributed to the curcumin molecules encapsulated inside the core-shell microgels. To confirm that the microgels are not just distributed on the surface of cells, the Z-scanning confocal fluorescence transmission images were taken for the B16F10 cells (Figure 4–10), which indicate that the curcumin-trapped core-shell microgels indeed illuminate the entire cell, but mainly distributed in the cytoplasm and perinuclear region of the cells. It is clear that the B16F10 cells can readily phagocytose these small-sized core-shell microgels.
Figure 4–10 Z-Scanning confocal fluorescence transmission images of mouse melanoma cells B16F10 incubated with curcumin loaded core-shell microgels VEM3.

4.3.7 *In vitro* cytotoxicity

For future biological applications, especially clinical applications, material must be non-cytotoxic or low-cytotoxic. As shown in Figure 4–9, almost no signs of morphological damage to the cells were observed upon the treatment with microgels, demonstrating the minimal cytotoxicity of the microgels. To further evaluate the cytotoxicity of the empty microgels and to verify whether the released curcumin was still pharmacologically active, *in vitro* cytotoxicity
tests were elaborately conducted against B16F10 cells. The studied concentrations were set on the base of potential drug loading capacity of microgels in order to compare the cytotoxicity of the empty microgels with the drug-loaded ones. Figure 4-11 shows that the empty microgels were non-cytotoxic to B16F10 cells in concentrations of up to 428 µg/mL and 480 µg/mL for VEM1 and VEM4, respectively. These results indicate that our microgels based on the PVAS core and nonlinear PEG gel shell exhibit an excellent *in vitro* biocompatibility. In contrast, when the cells were incubated with curcumin-loaded microgels, the cell viability drastically decreased even at a concentration as low as 143.7 µg/mL and 106.9 µg/mL for VEM1 and VEM4, respectively (equivalent to about 40.7 µg/mL free curcumin in both systems). These results indicate that the curcumin-loaded microgels provide high anticancer activity. The lower cytotoxicity of curcumin-loaded microgels than the free curcumin solutions is reasonable due to the sustained release property of the curcumin-loaded microgels and thus the lower free drug concentration.
Figure 4–11 Comparison of B16F10 cell survivability following treatments with core-shell microgels VEM1 and VEM4 as drug carriers, respectively; (●: curcumin-loaded VEM1, ■: curcumin-loaded VEM4). B16F10 cell survivability after treated with free microgels (○: VEM1; □: VEM4) and free curcumin solutions (♦).

4.4 Conclusion

Well-defined thermo-responsive PVAS@PEG core-shell microgels with the PVAS microgels as hydrophobic core and the nonlinear PEG gel as a hydrophilic shell could be successfully synthesized via precipitation polymerization. The rationally designed PVAS core chain networks can effectively store the hydrophobic curcumin drug molecules via hydrophobic interactions, thus provide high drug loading capacity. The nonlinear PEG gel shell composed of the copolymer P(MEO₂MA-co-MEO₅MA) with the comonomer ratio of MEO₂MA:MEO₅MA = 1:2 could not only increase the stability of the core-shell microgels dispersion in aqueous media,
but also prevent the PVAS hydrophobic core network from collapsing. This designed structure of hydrophobic core with a large mesh size in the core-shell microgels can load hydrophobic drug molecules much more effectively, which indicates that both the drug-core hydrophobic interactions and the mesh size of core networks are important to determine the drug loading capacity. The thermo-sensitive nonlinear PEG gel shell of the microgels could trigger the drug release by local temperature change, offering sustained drug release profiles. Although curcumin was selected as a specific model drug in this text, the core-shell nanostructured microgels can be generalized to deliver many other types of poor soluble or instable therapeutic agents.
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4.5 References


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Chapter 5

Water-dispersible Core-Shell Structured Magnetic-Polymer Hybrid Nanogels for Curcumin Delivery

5.1 Introduction

Magnetic nanoparticles (NPs) have attracted considerable attention in the past because of their exclusive advantage for targeted drug delivery, magnetic resonance imaging (MRI), bioseparation, biosensor, and hyperthermia\(^1\)\(^-\)\(^5\). Superparamagnetic iron oxide NPs (e.g. maghemite Fe\(_3\)O\(_4\)), as the typical magnetic NPs appear to be quite suitable for a drug targeted carrier, due to their capability of delivering pharmaceuticals to a specific site of the body by means of a gradient magnetic field\(^6\)\(^-\)\(^8\). However, bare superparamagnetic iron oxide NPs are toxic and very easy to aggregate. The surface modifications of the as-prepared Fe\(_3\)O\(_4\) NPs by polymers, proteins, or other biomolecules were carried out to achieve their biomedical applications. Biopolymer-coated magnetic NPs would be more suitable as drug carriers, because of their high physiological stability, biocompatibility, bio/magnetic targeting, and therapeutic functionalities\(^9\)\(^-\)\(^11\). Recently, many studies have focused on the fabrication of versatile core-shell structured magnetic-polymer composites\(^12\)\(^-\)\(^14\). However, to our knowledge, no polymer-coated
magnetic NPs have been specifically designed to deliver curcumin molecules, a traditional anti-tumor drug.

Curcumin is a yellow compound presented in spice turmeric (Curcuma longa), which can prevent carcinogen-induced tumorigenesis and inhibit growth of many tumor cell lines. Unfortunately, its exceedingly low water solubility, rapid metabolism in body, and low oral bioavailability has hindered clinical advancement to bring this safe and affordable natural product to the forefront against cancers and other chronic diseases. It is expected that the polymer-grafted magnetic NPs as drug delivery carriers can be guided and concentrated in anticipated target site by application of an external magnetic field, consequently minimize severe side effects and enhance the therapeutic effects of drugs. On the other hand, the responsive polymer shell that can undergo stimuli-responsive volume phase transitions should trigger the drug release in response to environmental stimuli, such as pH, temperature, light, and electrical field change. Among these smart materials, magnetic NPs coated with temperature-sensitive nanogels have been studied for controlled and targeted drug releasing systems. After guiding nanogels to a disease site by external magnetic field, these smart hybrid nanogels can efficiently penetrate into individual cells by encapsulation, surface attachment or entrapment due to their small size. When temperature increases, the nanogel will change the swelling degree and release out the drugs, resulting in an efficient drug accumulation at the targeted site. So far, magnetic NPs coated with poly(N-isopropylacrylamide) (PNIPAM) were most extensively studied because of their responsiveness to temperature stimuli and enhanced drug-loading ability. However, the well-studied PNIPAM-coated magnetic NPs have not been translated into a biomedical breakthrough due to the general concerns on the toxicity of NIPAM monomers. To meet the general requirement in biocompatibility of the materials used for drug delivery systems, one
important challenge is to develop biocompatible thermo-responsive polymers. The recently developed nonlinear poly(ethylene glycol) (PEG) polymers containing short oligo(ethylene glycol) side chains have attracted tremendous interests for the construction of polymeric drug carriers and other biomaterials applications due to their thermo-sensitive, water-soluble, and biocompatible properties. Another advantage of this new type of nonlinear PEG polymers is that their lower critical solution temperature (LCST) in aqueous solution is tunable through the control in the compositions of the oligo(ethylene glycol) side chains. The longer the oligo(ethylene glycol) side chains, the higher the LCST of the resultant nonlinear PEG polymers.

**Scheme 5–1** Schematic illustration of curcumin-loaded Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels with small Fe₃O₄ NP as core embedded in the poly(4-allylanisole)-PEG double-layer gel shell. The hydrophobic curcumin drug is mainly encapsulated within the inner hydrophobic poly(4-allylanisole) shell, which is coated by an outer PEG-based shell to offer biocompatibility, stability in aqueous media, and temperature responsiveness. When temperature increases, the hybrid nanogels shrink and the loaded curcumin molecules are released out.

In this work, we design a novel type of magnetic core-shell structured hybrid nanogel for highly efficient curcumin delivery carriers. As shown in Scheme 5–1, the newly designed core-shell hybrid nanogels is composed of a magnetic Fe₃O₄ NP as center core, a hydrophobic poly(4-
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allylanisole) gel layer as inner shell, and a hydrophilic, biocompatible, and thermo-responsive nonlinear PEG gel as outer shell. The magnetite Fe₃O₄ NPs are designed to provide the hybrid nanogels with fast magnetic response, magnetic site-targeting ability and magnetic resonance imaging function. The inner poly(4-allylanisole) gel shell is particularly designed to improve the drug loading yields for the very hydrophobic and delicate curcumin molecules. 4-Allylanisole is a natural organic compound produced by many conifers and herbs. It is usually used in the preparation of fragrances and a flavoring additive of bakery products, both alcoholic and nonalcoholic beverages, chewing gum, confections, fish, ice cream, salad, sauces, and vinegar. Considering its structural similarity to the structural units of curcumin molecules, we expect that the poly(4-allylanisole) gel network chains can interact with curcumin molecules effectively through the π-π stacking and other hydrophobic interactions, thus enhance the curcumin drug loading capacity significantly. The outer nonlinear PEG gel shell is designed to provide the water dispersibility and biocompatibility for the hybrid nanogels as well as the thermo-triggerable drug release. The nonlinear PEG gel shell can be synthesized by polymerizing and crosslinking the comonomers of 2-(2-methoxyethoxy)ethyl methacrylate (MEO₂MA) and oligo(ethylene glycol) methyl ether methacrylate (Mₙ = 300 g/mol, MEO₅MA) at a molar ratio of 1:2. Our previous reports proved that this rational ratio of PEG comonomers can lead the resultant nonlinear PEG nanogel to a continuous thermo-responsive volume phase transition with tunable slopes across the physiologically important temperature range of 37–42 °C, which are typical abnormal temperature range found in many pathological zones such as tumors. Table 5–1 display chemical structures of all the monomers, crosslinkers, and curcumin molecules. For drug carrier applications, the size of the hybrid nanogels is another critical parameter. In our design, the core-shell-shell hybrid nanogels can be synthesized by precipitation polymerization method, thus the
inner/outer polymer gel shell thickness and the overall size of the hybrid nanogels can be well controlled through the feeding ratios of shell precursors to the core template NPs amount during synthesis. The effects of inner and outer shell thickness on the magnetic properties, drug loading capacity, sustained drug release behavior, and cytotoxicity of the drug-free and drug-load hybrid nanogels will be evaluated. The combination of the characteristic functions from the each component designed in the hybrid nanogels would enable the magnetic hybrid nanogels to become a highly efficient smart curcumin delivery carrier with magnetic site-targeting ability, magnetic resonance imaging contrasting ability, high curcumin loading capacity, triggerable sustained release, good cell penetration ability, and high therapeutic efficacy.
Table 5-1 Structures and function of all the monomers, crosslinkers, and curcumin molecules using in this work.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure" /></td>
<td>4-Allylanisole</td>
<td>Major monomer Curcumin loading component</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure" /></td>
<td>2-(2-methoxyethoxy)ethyl methacrylate (MEO2MA)</td>
<td>Major monomer Thermo-responsive component</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure" /></td>
<td>Oligo(ethylene glycol)methyl ether ethacrylate (MEO5MA)</td>
<td>Major monomer Thermo-responsive component</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure" /></td>
<td>Divinylbenzene (DVB)</td>
<td>Crosslinker</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure" /></td>
<td>Poly(ethylene glycol) dimethacrylate (PEGDMA)</td>
<td>Crosslinker</td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure" /></td>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Surfactant</td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure" /></td>
<td>2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH)</td>
<td>Initiator</td>
</tr>
<tr>
<td><img src="image8.png" alt="Structure" /></td>
<td>Curcumin</td>
<td>Cancer Drug</td>
</tr>
</tbody>
</table>

5.2 Experimental

5.2.1 Materials

All chemicals were purchased from Aldrich. 2-(2-methoxyethoxy)ethyl methacrylate (MEO2MA, 95%), oligo(ethylene glycol)methyl ether methacrylate (MEO5MA, $M_n = 300$ g/mol) and poly(ethylene glycol) dimethacrylate (PEGDMA, $M_n \approx 550$ g/mol) were purified with
neutral Al₂O₃. Curcumin was purified with anhydrous ethanol. Iron (III) chloride (FeCl₃), Iron (II) chloride tetrahydrate (FeCl₂⋅4H₂O), 4-allylanisole, divinylbenzene (DVB), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium dodecyl sulfate (SDS), anhydrous ethanol, Dulbecco's Modified Eagle Medium (DMEM), and Fetal bovine serum (FBS) were used as received without further purification. The water used in all experiments was of Millipore Milli-Q grade.

5.2.2 Synthesis of Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels

5.2.2.1 Synthesis of Fe₃O₄ nanoparticles

In a 250 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, FeCl₃ (5 mM), FeCl₂⋅4H₂O (2.5 mM), and 30 mL distilled water were heated to 80 °C. Then 5 mL of NaOH (10 M) were added. The reaction was allowed to proceed for 1 h. The solution was centrifuged at 6000 rpm (20 min, Thermo Electron Co. SORVALL® RC-6 PLUS superspeed centrifuge) with the supernatant discarded and the precipitate was redispersed in 30 mL deionized water. SDS-stabilized Fe₃O₄ nanoparticles were obtained by adding 4.6 × 10⁻⁴ mol SDS into 0.125 mL aqueous solution of Fe₃O₄ nanoparticles and 100 mL distilled water. The mixture was stirred and aged overnight under N₂ atmosphere.

5.2.2.2 Synthesis of Fe₃O₄@poly(4-allylanisole) nanogels

The Fe₃O₄@poly(4-allylanisole) nanogels were prepared according to the recipes listed in Table 5–2. In a 250 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, 100 mL as-prepared aqueous solution of SDS-stabilized Fe₃O₄ NPs was heated to 30 °C, followed by addition of 4-allylanisole monomer and DVB crosslinker under stirring.
Different feeding compositions for the synthesis of the Fe$_3$O$_4$@poly(4-allylanisole) hybrid nanogels are listed in Table 5--2 to control the different inner shell thickness. After 30 min, the temperature was raised to 70 °C and polymerization was initiated by adding 1 mL of 0.105 M AAPH. The polymerization was allowed to react for 5 h. The resulted suspension was centrifuged three times at 10,000 rpm (30 min, Thermo Electron Co. SORVALL® RC-6 PLUS super-speed centrifuge) with supernatant discarded and the precipitate was redispersed in 100 mL deionized water. The resultant Fe$_3$O$_4$@poly(4-allylanisole) nanogels were used as template particles for subsequent precipitation polymerization to add the outer shell of nonlinear PEG copolymer gel of P(MEO$_2$MA-co-MEO$_3$MA). The Fe$_3$O$_4$@poly(4-allylanisole) nanogels are coded as FA.
Table 5–2

Feeding compositions for synthesis and hydrodynamic radius ($R_h$) of the Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inner Shell Solution (mmol)</th>
<th>Outer Shell Solution (mmol)</th>
<th>$R_h^a$ (nm)</th>
<th>Outer Shell Thickness$^b$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Allylanisole</td>
<td>0.594</td>
<td>5.61 $\times$ 10$^{-2}$</td>
<td>97.5</td>
<td></td>
</tr>
<tr>
<td>FAE–1</td>
<td>0.594</td>
<td>5.61 $\times$ 10$^{-2}$</td>
<td>0.75</td>
<td>1.50</td>
</tr>
<tr>
<td>FAE–2</td>
<td>0.594</td>
<td>5.61 $\times$ 10$^{-2}$</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>FAE–3</td>
<td>0.594</td>
<td>5.61 $\times$ 10$^{-2}$</td>
<td>0.25</td>
<td>0.50</td>
</tr>
<tr>
<td>FA–2</td>
<td>0.891</td>
<td>8.42 $\times$ 10$^{-2}$</td>
<td>0.75</td>
<td>1.50</td>
</tr>
</tbody>
</table>

$^a$ Hydrodynamic Radius ($R_h$) measured by Dynamic light scattering (DLS) method at 18 °C and a scattering angle of $\theta = 30^\circ$.

$^b$ Outer shell thickness is equal to $R_h$ of Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels (FAE) subtracted by $R_h$ of corresponding Fe$_3$O$_4$@poly(4-allylanisole) nanogels (FA).

5. 2. 2. 3 Synthesis of Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels

The outer shell precursors of MEO$_2$MA and MEO$_5$MA comonomers mixture in 1:2 molar ratio and PEGDMA crosslinker were dissolved into 100 mL purified Fe$_3$O$_4$@poly(4-allylanisole) nanogels dispersion. The mixture was heated to 70 °C under a N$_2$ purge. After 30 min, 1 mL of AAPH (0.105 M) initiator was added to start the polymerization. The synthesis was allowed to proceed to total 5 h. The resulted Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels were
purified with centrifugation/redispersion in water for three cycles, followed by 3 days of dialysis (Spectra/Pro® molecular porous membrane tubing, cutoff 12,000–14,000) against very frequently changed water at room temperature (~22 °C). Different feeding compositions for the synthesis of the Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels are listed in Table 5–2 to control the different outer shell thickness. The Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels are coded as FAE.

5.2.3 In vitro magnetic manipulation

15 mL of the hybrid nanogel dispersions of different pH values were placed in $\varnothing 25 \times H45$ mm bottles, respectively. A permanent magnet was placed next to the bottle to provide a magnetic field of 0.1 T. The hybrid nanogel dispersions were sampled in the center region of the volume at defined time period and assayed by UV-vis spectrometry at 440 nm.

5.2.4 Curcumin loading and release

Curcumin was loaded into the porous hybrid nanogels mainly driven by hydrophobic interactions. 5 mL Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels dispersion was stirred in an ice water bath for 30 min. 4 mL fresh curcumin solution of 1mg/mL in anhydrous ethanol was then added dropwise to the vial. After stirring overnight, the suspension was centrifuged at 10,000 rpm for 15 min at 22 °C. To remove free curcumin, the precipitate was redispersed in 5 mL water, and further purified by repeated centrifugation and washing for at least six times. All the upper clear solutions were collected, and the concentration of free curcumin was determined by UV-vis spectrometry at 435 nm. The emission intensity at 566 nm with the excitation wavelength of 420 nm on the upper clear solutions was also recorded to confirm the loading.
amount. Optical signal was converted to concentration based on the linear calibration curve with \( R^2 > 0.99 \) measured using the curcumin solution with known concentrations under the same condition. The amount of loaded curcumin in the hybrid nanogels was calculated by deducting the total curcumin amount in upper clear solution from the total curcumin amount (4 mg) in the initial solution. The loading capacity is expressed as the mass of loaded drug per unit weight of dried hybrid nanogels.

The *in vitro* release test of curcumin from the hybrid nanogels was evaluated by the dialysis method. The curcumin-loaded nanogel dispersion was diluted to 0.15 mg/mL for the release experiments. A dialysis bag (Spectra/Pro® molecularporous membrane tubing, cutoff 12,000–14,000) filled with 1 mL diluted curcumin-loaded hybrid nanogels was immersed in 50 mL 0.005 M phosphate buffer solutions (PBS) of pH = 6.15 at different temperatures. The released curcumin outside of the dialysis bag was sampled at defined time period and assayed by fluorescence spectrophotometer at 566 nm upon excitation at 420 nm. Cumulative release is expressed as the total percentage of drug released through the dialysis membrane over time.

### 5.2.5 *In vitro* cytotoxicity

B16F10 cells (2000 cell/well) were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin in a 96-well plate, and exposed to hybrid nanogels FAE–(1–4) respectively. To cover the high concentrations, the hybrid nanogels were concentrated and adjusted to an appropriate concentration in DMEM right before feeding into the wells. The plate was incubated at 37 °C for 24 h. The medium was then aspirated, and these wells were washed using fresh serum-free DMEM. After that, 25 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) were added to the wells. After
incubation for 2 h, the solution was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to each well, and the plate was sealed and incubated for 30 min at 37 °C with gentle mixing. Three portions of the solution obtained from each well were transferred to three respective well of a 96-well plate. Cell viability was measured using a microplate reader at 570 nm. Positive controls contained no nanogels, and negative controls contained MTT.

5.2.6 Characterization

The PL spectra were obtained on a JOBIN YVON Co. FluoroMax®-3 Spectrofluorometer equipped with a Hamamatsu R928P photomultiplier tube, calibrated photodiode for excitation reference correction from 200 to 980 nm, and an integration time of 1 s. The morphology of the hybrid nanogels was characterized with transmission electron microscopy (TEM). The TEM images were taken on a FEI TECNAI transmission electron microscope at an accelerating voltage of 120 kV. Approximately 10 µL of diluted hybrid nanogel suspension was dropped on a Formvar-covered copper grid (300 meshes) and then air-dried at room temperature for the TEM measurements. The pH values were obtained on a METTLER TOLEDO SevenEasy pH meter.

Dynamic light scattering (DLS) was performed on a standard laser light scattering spectrometer (BI-200SM) equipped with a BI-9000 AT digital time correlator (Brookhaven Instruments, Inc.). A He-Ne laser (35 mW, 633 nm) was used as the light source. All nanogel solutions were passed through Millipore Millex-HV filter with a pore size of 0.80 µm to remove dust before the DLS measurements. In DLS, the Laplace inversion of each measured intensity-intensity time correlated function can result in a characteristic line width distribution $G(\Gamma)$. For a purely diffusive relaxation, $\Gamma$ is related to the translational diffusion coefficient $D$ by $(\Gamma/q^2)_{q \to 0, \Gamma \to 0} = D$, where $q = (4\pi n/\lambda)\sin(\theta/2)$ with $n$, $\lambda$, and $\theta$ being the solvent refractive index,
the wavelength of the incident light \textit{in vacuo}, and the scattering angle, respectively. \( G(I) \) can be further converted to a hydrodynamic radius \( (R_h) \) distribution by using the Stokes-Einstein equation, \( R_h = (k_BT/(6\pi\eta))D^{-1} \), where \( k_B \), \( T \), and \( \eta \) are the Boltzmann constant, the absolute temperature, and the solvent viscosity, respectively.

5.3 Results and Discussion

5.3.1 Synthesis of Fe\(_3\)O\(_4@\)poly(4-allylanisole)-PEG hybrid nanogels

The synthesis strategy of the hybrid nanogels with hydrophobic-hydrophilic double-layer polymer gel as shell and Fe\(_3\)O\(_4\) NPs as core involves the first synthesis of Fe\(_3\)O\(_4\) NPs as core template, followed by coating the hydrophobic inner poly(4-allylanisole) gel shell and then the hydrophilic thermo-responsive nonlinear PEG gel outer layer, respectively. The magnetite (Fe\(_3\)O\(_4\)) NPs were prepared by the coprecipitation of Fe(II) and Fe(III) salts with a molecular ratio of 1:2 in aqueous solutions at pH = 11−12 under N\(_2\) for 1 h\(^{40}\). The reaction step is as follows:

\[
1 \text{ mol FeCl}_2 + 2 \text{ mol FeCl}_3 \xrightarrow{\text{NaOH}} \text{Fe}_3\text{O}_4
\]

The resulting black product was isolated by centrifugation (6000 rpm, 20 min) with the supernatant discarded and the precipitate was redispersed in 30 mL deionized water. In order to stabilize the Fe\(_3\)O\(_4\) NPs dispersed in aqueous solution, the SDS surfactant molecules were added to cap the Fe\(_3\)O\(_4\) NPs. The SDS molecules might assemble to form a bi-layer at the surface of Fe\(_3\)O\(_4\) NPs\(^4\). The first monolayer binds with the anionic head groups pointed down toward the surface of Fe\(_3\)O\(_4\) NPs. The exposure of the alkyl chains to the aqueous solvent is energetically unfavorable, resulting in the adsorption of a second surfactant layer with the SDS’s head groups
facing toward the water. In turn, the bi-layer capping of SDS molecules would inhibit the aggregation and provide additional stability for Fe$_3$O$_4$ NPs.

With the SDS-stabilized Fe$_3$O$_4$ NPs as core template, the polymer gel shell can be added through the precipitation polymerization. After the addition of initiator, the 4-allylanisole monomers will start to polymerize and grow into poly(4-allylanisole) segments, which are very hydrophobic and will precipitate into the hydrophobic area of the SDS-capped Fe$_3$O$_4$ NPs. The continuous polymerization and crosslinking of the 4-allylanisole monomers will lead more and more poly(4-allylanisole) segments deposited into the hydrophobic zone and thus increase the thickness of the poly(4-allylanisole) gel layer until all the monomers are polymerized. The resulting SDS-stabilized poly(4-allylanisole)-coated Fe$_3$O$_4$ NPs can be continuously used as a seed for the subsequent precipitation polymerization to coat the thermo-responsive P(MEO$_2$MA-co-MEO$_5$MA) copolymer gel outer layer. Although the comonomers of the MEO$_2$MA and MEO$_5$MA are quite water soluble, their corresponding polymers of P(MEO$_2$MA) and P(MEO$_5$MA) exhibit a lower critical solution temperature (LCST) around 20 and 61 °C, respectively$^{41}$. A proper control on the feeding ratio of these two PEG oligomer-based monomers can tune the LCST of their copolymer into the physiologically important temperature range$^{42}$. Therefore, at the synthetic temperature (70 °C) above the LCST of the copolymer, the resultant P(MEO$_2$MA-co-MEO$_5$MA) copolymer segments are hydrophobic and will precipitate into the hydrophobic zone of the SDS-stabilized poly(4-allylanisole)-coated Fe$_3$O$_4$ NPs, resulting a core-shell-shell structured hybrid nanogel. At the physiological temperature range or below, the outer P(MEO$_2$MA-co-MEO$_5$MA) copolymer gel layer is hydrophilic, thus enable the resultant Fe$_3$O$_4$@poly(4-allylanisole)-PEG core-shell-shell hybrid nanogels being dispersed in aqueous solution very well. The TEM image of the Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels
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(FAE−1) shown in Figure 5−1 confirms that the resulted hybrid nanogels possess a spherical morphology and an uniform distribution of particle size. A boundary between a dark core and a light contrast shell can be clearly observed. The high contrast of core with ca. 10 ± 3 nm in diameter is due to the markedly high electron density of Fe₃O₄ NPs, while the low contrast of the thick shell is owing to the polymer gel layers of poly(4-allylanisole) and P(MEO₂MA-co-MEO₅MA) copolymers. Furthermore, a careful view on the magnified image of the FAE−1 hybrid nanogel particle, shown in the inset of Figure 5−1, indicates a clear boundary between the inner and outer polymer gel layers, confirming the double-layer polymer gel shell structure. The contrast distinction between the double-layer shell is associated with the electron density difference of the poly(4-allylanisole) gel and P(MEO₂MA-co-MEO₅MA) copolymer gels. At the synthetic temperature of 70 °C, the inner crosslinked poly(4-allylanisole) chain networks are very hydrophobic and densely packed, which can hinder the newly deposited nonlinear PEG-based chain segments penetrating into the inner shell area, resulting an outer gel shell mainly from the nonlinear PEG copolymer network chains⁴³.

**Figure 5−1** Typical TEM images of Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels FAE−1.
For drug delivery carriers, it is very important that the size and polymer shell thickness of the hybrid nanogels can be controlled in synthesis to meet the application needs. In our synthetic strategy, the simple change in the feeding ratios of the shell monomers to the template core NPs can control the thickness of the polymer gel shell. As shown in Table 5–2, with the same amount of Fe₃O₄ NPs as core template, the increase in the feeding of 4-allylanisole monomer and DVB crosslinker by 50% can significantly increase the poly(4-allylanisole) gel shell thickness by 40 nm (Rₜ values for FA-1 and FA-2 are 97.5 and 136.5 nm, respectively). Figure 5–2 shows the size distributions, in terms of the Rₜ measured at T = 24 °C and θ = 30°, of the Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels FAE–(1–4) synthesized under different feeding ratios of shell monomers to core template NPs (See Table 5–2). First, these resultant Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels have a very narrow size distribution with a polydispersity index of \( \mu_2/\langle I \rangle^2 = 0.005 \). Second, the hybrid nanogels FAE–(1–3), synthesized using the same amount of Fe₃O₄@poly(4-allylanisole) NPs (FA–1) as core template but with the gradually decreased feeding amount of shell precursors of MEO₂MA and MEO₅MA, exhibit a gradually decreased size and outer shell thickness with the Rₜ being 166.8 nm, 144.7 nm, and 136.6 nm, respectively. Clearly, if we use the same core template NPs, the shell thickness can be tuned by a simple change in the feeding amount of shell precursors. On the other hand, the results shown in Table 5–2 indicate that the shell thickness can also be controlled by the size of core template NPs. For example, when we use the same amount of nonlinear PEG shell precursors but larger Fe₃O₄@poly(4-allylanisole) NPs (FA–2) as core template, the outer shell is thinner than that obtained with FA–1 NPs as core template.
**Figure 5–2** Hydrodynamic radius distributions of Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels FAE−1 (■), FAE−2 (●), FAE−3 (▲), and FAE−4 (▼), measured at 24 °C and a scattering angle of θ = 30°.

**5. 3. 2** Temperature induced volume phase transition

**Figure 5–3** Temperature dependence of the average $R_h$ values of Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels FAE−1 (■), FAE−2 (●), FAE−3 (▲), and FAE−4 (▼), measured at a scattering angle θ = 30°.
Figure 5–3 shows the temperature-induced volume phase transition of the diluted Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels dispersions (∼5.0 × 10⁻³ wt%) in PBS solution (0.005 M, pH = 7.4), in terms of the change of $R_h$ measured at a scattering angle of $\theta = 30^\circ$. It is clear that the increase in temperature can lead to a significant decrease in the size of the hybrid nanogels. The driving force for such a temperature-induced volume phase transition is believed to be a subtle balance between the ability of the polymer to form hydrogen bonds with water and the inter-/intra-molecular hydrophobic forces. In our studied temperature range, the crosslinked poly(4-allylanisole) chain networks are very hydrophobic and exhibit no conformational response to the external stimulus of temperature change. The observed temperature-induced volume phase transition of the hybrid nanogels should be contributed from the outer nonlinear PEG gel shell composed of P(MEO₂MA-co-MEO₅MA) copolymer chain networks. The formation of hydrogen-bonds between the ether oxygens of the side PEG chains and the hydrogen of water is generally considered to be one of the key factors responsible for the unusual water solubility of this type of nonlinear PEG polymers. On the other hand, the hydrophobicity of the apolar backbone would counterbalance this favorable effect in water. The proper feeding ratio of 1:2 between the two monomers of MEO₂MA and MEO₅MA can lead to a volume phase transition region across the physiologically important temperature range (37–42 °C), which are typical abnormal temperature range found in many pathological zones such as tumor.

It should be noted that the diluted Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogel dispersions (∼ 5.0 × 10⁻³ wt%) are highly stable without aggregation in the studied temperature range of 24–45 °C, as revealed by the single narrow $R_h$ distribution peak with the polydispersity index $\mu_2/\langle I \rangle^2 \leq 0.005$ (Figure 5–4). No sediment was observed in the hybrid nanogel solutions.
even after a few months. The excellent stability of the hybrid nanogels in water is very important to serve as a drug carrier. Considering that most of the anti-cancer drug molecules are very hydrophobic, our newly designed hydrophobic-hydrophilic double shell structured hybrid nanogels should be desirable for anti-cancer drug carriers. While the inner hydrophobic poly(4-allylanisole) inner shell can provide high drug loading capacity for the hydrophobic anti-cancer drugs, the hydrophilic outer PEG-based gel shell can enable the hybrid nanogels to be dispersed well in physiological medium and further penetrate into the cells. Furthermore, the thermo-responsive nonlinear PEG outer shell can control the release of the drug molecules under a temperature stimulus.

![Diagram](image.png)

**Figure 5–4** Size distribution of Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels FAE–1 at different temperatures (■: 24 °C; ●: 33 °C; ▲:37.5 °C; and ▼: 45 °C). All measurements were made at pH = 7.4 and a scattering angle of $\theta = 30^\circ$. 

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5. 3. 3 Magnetic properties of the Fe$_3$O$_4$@poly(4-allylanisole) and Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels

![Figure 5-5](image)

**Figure 5–5** Typical digital photographs of the Fe$_3$O$_4$@poly(4-allylanisole) hybrid nanogels (FA–1, A) and Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels (FAE–1, B) before and after placing a permanent magnet next to it. The FAE–1 hybrid nanogels can also be easily redispersed into water again after the removal of the external magnet and a gentle shaking (right photo in B).
Figure 5–5 shows the digital photographs of the Fe₃O₄@poly(4-allylanisole) hybrid nanogels (FA–1, A) and Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels (FAE–1, B) before and after placing a permanent magnet next to it. The magnetic hybrid nanogels are uniformly dispersed in water with no visible aggregates or precipitates formed. The hybrid nanogel particles can be quickly separated and collected from the aqueous suspension under the 0.1 T external magnetic field, demonstrating that the FAE–1 hybrid nanogels possess a quick magnetic response. The FAE–1 hybrid nanogels can be easily redispersed into water again after the removal of the external magnet and a gentle shaking (right photo in B). The quick magnetic response of these Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels provides advantages as drug carriers for separation and magnetic-targeted on site drug delivery.

![Hysteresis Loop](image)

**Figure 5–6** Typical hysteresis loop measured at 300 K of the Fe₃O₄ core NPs, Fe₃O₄@poly(4-allylanisole) hybrid nanogels (FA–1), and Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels (FAE–1).
Zero-field-cooling (ZFC) and field-cooling (FC) curves of the Fe$_3$O$_4$ core NPs, Fe$_3$O$_4$@poly(4-allylanisole) hybrid nanogels (FA$^{-}$1), and Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels (FAE$^{-}$1), respectively, measured in an applied field of 50 Oe.

The Fe$_3$O$_4$@poly(4-allylanisole)-PEG hybrid nanogels possess the magnetic properties from the Fe$_3$O$_4$ NP cores. While a detailed analysis of the magnetic properties is out of the scope of this project, we show in Figure 5–6 the magnetization cycles of the dried hybrid nanogels recorded at 300 K in an applied magnetic field up to 40 000 Oe. The loop presents hysteresis, revealing the ferromagnetic nature of the hybrid nanogels$^{45}$. The results for the hard core Fe$_3$O$_4$ NPs and the Fe$_3$O$_4$@poly(4-allylanisole) nanogels (FA$^{-}$1) are also presented for comparison. Obviously, the growth of poly(4-allylanisole) gel shell onto the Fe$_3$O$_4$ NP templates led to a significant decrease in the values of saturation magnetization ($M_s$) and remnant magnetization ($M_r$). The addition of the outer nonlinear PEG gel shell further decreases the $M_s$ and $M_r$ values (FAE$^{-}$1 vs. FA$^{-}$1). The reason should be attributed to the significant decrease in the content of magnetite Fe$_3$O$_4$ NPs in the hybrid nanogels in terms of the magnetization per gram of samples.
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The temperature dependence of zero-field-cooled (ZFC) and field-cooled (FC) magnetization for the Fe₃O₄ NPs, FA–1 and FAE–1 are presented in Figure 5–7. ZFC and FC curves separate from each other from 5 to 325 K, and no transition from ferromagnetism to superparamagnetism can be observed. These results further confirm the ferromagnetism of the hybrid nanogels at room temperature. The ferromagnetism of the hybrid nanogels could provide the contrast ability for magnetic resonance imaging function.

5.3.4 Drug loading capacity of the Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels

Curcumin is poorly soluble in water at neutral pH with the macroscopic undissolved flakes visible in the solution. It also has been determined that the half-time for the hydrolytic degradation of curcumin in aqueous solution containing 10% organic solvent at pH ≈ 6, 7, and 8 is 4.2 × 10³ h, 15 h and 3.5 × 10⁻² h, respectively. Thus curcumin would undergo rapid hydrolytic degradation at neutral or basic pH values, resulting in the loss of pharmaceutical activities. In order to avoid the curcumin molecular degradation, we loaded the curcumin drug into the Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels in aqueous dispersion at pH = 5.7. Since the poly(4-allylanisole) has similar structural units to the curcumin molecules, we expect that the curcumin molecules diffused into the gel networks should have hydrophobic and π–π interactions with the poly(4-allylanisole) chains, thus curcumin molecules can build up in the interior of the poly(4-allylanisole) gel networks, resulting in high drug loading content.

Table 5–3. Curcumin loading capacity of the Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels

<table>
<thead>
<tr>
<th>Samples</th>
<th>FAE-1</th>
<th>FAE-2</th>
<th>FAE-3</th>
<th>FAE-4</th>
<th>Fe₃O₄@poly(4-allylanisole)</th>
<th>Fe₃O₄@nonlinear PEG hybrid nanogels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin loading capacity (w/w%)</td>
<td>35.3%</td>
<td>32.1%</td>
<td>30.5%</td>
<td>37.8%</td>
<td>20.3%</td>
<td>1.74%</td>
</tr>
</tbody>
</table>
Chapter 5

Table 5.3 compares the loading capacity of the hybrid nanogels FAE−(1–4) for curcumin drugs. Two features should be noted. First, the thicker the inner poly(4-allylanisole) gel layer, the higher the loading capacity of the resultant hybrid nanogels. As shown in Table 5–2, the hybrid nanogels of FAE−1 and FAE−4 contains the same amount of outer nonlinear PEG shell components, but FAE−4 has thinner PEG outer shell and thicker inner poly(4-allylanisole) gel shell compared to FAE−1. The results in Table 5–3 indicate that FAE−4 hybrid nanogels actually exhibits higher drug loading capacity for curcumin (37.8 wt%) than the FAE−1 does (35.3 wt%). When the outer PEG gel shell has a similar thickness (37.5 nm for FAE−4 and 39.2 nm for FAE−3), the effect of the inner poly(4-allylanisole) gel shell thickness on the curcumin loading content is even more significant. For example, the FAE−4 with thicker inner poly(4-allylanisole) gel shell demonstrate much higher curcumin loading capacity (37.5 wt%) in comparison with the FAE−3 hybrid nanogels with a curcumin loading content of 30.5 wt%.

Second, for the hybrid nanogels with the same thickness of inner poly(4-allylanisole) gel layer, the thickness of the hydrophilic nonlinear PEG gel outer shell could also affect the curcumin loading capacity. For example, the FAE−1, FAE−2, and FAE−3 hybrid nanogels have the same inner poly(4-allylanisole) gel thickness but a gradually decreased outer PEG gel shell thickness. The corresponding curcumin loading capacity for FAE−1 (35.3 wt%), FAE−2 (32.1 wt%), and FAE−3 (30.5 wt%) also demonstrates a gradually decreased trend. This result seems hard to understand because the hydrophilic nonlinear PEG chains in the outer shell should have weak interactions and should not be able to hold significant amount of hydrophobic curcumin molecules. In principle, the hydrophobic curcumin molecules should mainly stay in the inner hydrophobic poly(4-allylanisole) gel network due to the hydrophobic intermolecular complexation between the similar methoxy-aromatic structure of curcumin and anisole units.
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However, the gel network structures of the core and shell are dependent on each other in the core-shell nanostructured nanogels. The inner poly(4-allylanisole) gel shell is quite hydrophobic and densely shrunk at the studied temperatures, which limits the mesh size (or space) of the inner gel network to hold the hydrophobic curcumin molecules. The introduction of the hydrophilic and swollen outer PEG gel shell can restrict the inner poly(4-allylanisole) gel network from collapsing. Even though the inner poly(4-allylanisole) gel shell is hydrophobic and shrunk, the swollen PEG shell can pull up and stretch the inner poly(4-allylanisole) network, resulting in a bigger mesh size. The open network with larger mesh size of the inner poly(4-allylanisole) gel shell could hold more hydrophobic curcumin molecules. The thicker the swollen nonlinear PEG gel outer shell, the larger the pulling force to restrict the collapsing of the inner poly(4-allylanisole) gel network, thus the larger the mesh size and capacity of the inner gel shell to load the hydrophobic curcumin drug molecules.

Calculating from the approximate concentrations of the hybrid nanogels in aqueous dispersion, the loading capabilities are equivalent to 1.73, 1.57, 1.49, and 1.85 mM curcumin in water for FAE−1, FAE−2, FAE−3, and FAE−4, respectively. These concentrations of curcumin loaded in the hybrid nanogels are about two orders of magnitudes higher than the solubility of free curcumin in water at acidic or physiological pH (~ 20.1 µM)\textsuperscript{48, 49}. Furthermore, these hybrid nanogels can be further concentrated by a simple and fast magnetic separation, which makes it possible to realize a much higher curcumin dose. It should be noted that the curcumin-loaded hybrid nanogels are very stable in aqueous media. No sediment was observed after setting for a few weeks due to the high hydrophilicity of the outer PEG-based gel shell. Compared to the free curcumin molecules in aqueous media, the protection of the polymer gel network should also minimize the degradation of curcumin molecules.
5.3.5 *In vitro* release studies

![Graphs showing cumulative release vs. release time for different temperatures and nanogels.]

**Figure 5-8** Releasing profiles of curcumin from the Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels FAE–1 (A), FAE–2 (B), FAE–3 (C), and FAE–4 (D) at different temperatures. Curcumin has been pre-loaded into the nanogel at 22 °C. In the blank test (◊), 1 mL diluted solution of free curcumin (containing 5% ethanol) with an equivalent amount of drug trapped in FAE–1 was performed at 41°C. All releasing experiments were carried out in 50 mL PBS (0.005M) of pH = 6.15.
Figure 5–8 shows the release kinetics of curcumin from the FAE–(1–4) hybrid nanogels at different temperatures. The *in vitro* release test was performed in a PBS (pH = 6.15) to avoid the evident degradation of curcumin from the long time exposure in water. A free curcumin solution (containing 5% ethanol) with an equivalent amount of drug trapped in the hybrid nanogel FAE–1 was set as a blank release experiment, which shows that the dialysis membrane (cutoff 12000 – 14000 Da) played a negligible role in the release of curcumin. In comparison with the rapid curcumin release rate in the blank test under the same conditions, the release of curcumin from the hybrid nanogels demonstrates much slower rates, indicating a sustained curcumin release from the hybrid nanogels FAE–(1–4). More importantly, the release kinetics of curcumin from the hybrid nanogels significantly depends on the temperature of releasing medium. The increase in temperature can speed up the release of curcumin from all the four hybrid nanogels of FAE–(1–4). For example, after the same time period of 48 h, the amount of curcumin released from the FAE–1 hybrid nanogels increases from only 7.5 % at 22 °C to 31.6 % (37 °C), 52.1 % (39 °C), and 69.0 % (41 °C), respectively (Figure 5–8 A). The observed temperature dependency of the curcumin releasing rate should be associated with the thermo-sensitive outer shell of the nonlinear PEG-based gel. The increase in temperature induces a shrinking of the outer PEG gel shell. The shrunk and thinner outer PEG gel shell will not only compress the inner poly(4-allylanisole) gel network and thus squeeze out the curcumin molecules loaded in the interior of the gel network, but also reduce the restricted diffusion path length of the curcumin molecules from the inner gel layer to the medium outside the hybrid nanogel particles. Both effects can speed up the releasing rate of curcumin from the hybrid nanogels. Therefore, the higher the temperature is, the more the outer PEG gel network collapses, which leads to a faster releasing rate of curcumin from the nanogels. This hypothesis is further
confirmed by the effect of the outer shell thickness of nonlinear PEG gel on the releasing kinetics of curcumin from the hybrid nanogels. Curcumin molecules could be released more quickly from the hybrid nanogels coated with thinner outer PEG-based gel shell. For example, after the cumulative release for 48 h at the same temperature of 41 °C, 75.4 % and 78.3 % of loaded curcumin could be respectively released from the FAE-2 and FAE-3 hybrid nanogels with thin outer PEG gel shell. In contrast, only 69.0 % of curcumin could be released from the FAE−1 sample with the thickest outer PEG gel shell.

5. 3. 6  In vitro cytotoxicity

![Figure 5-9](image-url) Comparison of B16F10 cell viability following treatments with curcumin-loaded and drug free Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels of FAE−1 and FAE−4, respectively (■: curcumin-loaded FAE−1; ●: curcumin-loaded FAE−4; □: FAE−1; ○: FAE−4; ♦: free curcumin solutions).
Having demonstrated the curcumin delivery ability of the hybrid nanogels in buffer solution, we selected FAE−1 and FAE−4 hybrid nanogels to examine the *in vitro* cellular cytotoxicity of the drug free and curcumin-loaded hybrid nanogels. As shown in Figure 5−9, the drug free hybrid nanogels were non- or low-cytotoxic to B16F10 cells after incubation for 24 h even at high concentration of 706.0 and 659.2 µg/mL for FAE−1 and FAE−4, respectively, indicating an excellent *in vitro* biocompatibility benefited from the outer shell of nonlinear PEG gel. In contrast, the cell viability decreased to 36.4% and 27.5% for curcumin-loaded FAE−1 and FAE−4 hybrid nanogels, respectively, under the same conditions with the corresponding curcumin concentration of 249.2 µg/mL, indicating that the curcumin-loaded hybrid nanogels maintain the high anticancer activity of curcumin. These results should be attributed to the cellular uptake of these hybrid nanogels and the sustained release of curcumin from these hybrid nanogels. The cell viability of free curcumin solution in PBS (containing 5% ethonal) was 6.9% at the concentration of 249.2 µg/mL. The lower cytotoxicity of the curcumin-loaded hybrid nanogels than the free curcumin solutions with the equivalent curcumin concentration is understandable, because less than 30% of the loaded curcumin was released from the FAE−1 and FAE−4 hybrid nanogels in 24 h at 37 ºC.

5.4 Conclusion

Well-defined magnetic hybrid nanogels with a core-shell-shell nanostructure composed of the magnetic Fe₃O₄ NPs in the core, hydrophobic poly(4-allylanisole) gel as inner shell, and the hydrophilic thermo-responsive PEG gel as outer shell could be successfully synthesized via precipitation polymerization. The resultant Fe₃O₄@poly(4-allylanisole)-PEG hybrid nanogels demonstrate fast magnetic response for separation/collection and on-site magnetic targeting.
ability. The hybrid nanogels with ferromagnetic property could also be potentially used for magnetic resonance imaging contrast. The hydrophobic aromatic groups of the rationally designed poly(4-allylanisole) inner shell gel can form strong hydrophobic complexation with the curcumin molecules, resulting in high drug loading capacity. The polymer gel could also protect the curcumin molecules from degradation as in free aqueous solution. The outer shell of the nonlinear PEG gel composed of the P(MEO₂MA-co-MEO₅MA) copolymer network with the comonomer ratio of MEO₂MA:MEO₅MA = 1:2 could not only increase the stability and biocompatibility of the hybrid nanogels in aqueous media, but also trigger the curcumin drug release by local temperature stimuli in the physiologically important temperature range, offering sustained drug release profiles. While the drug-free hybrid nanogels are nontoxic to B16F10 tumor cells, the curcumin-loaded hybrid nanogels exhibit potent cytotoxicity against cell. This high therapeutic efficacy of the curcumin-loaded hybrid nanogels is likely to bring this natural product to the forefront of the therapeutic agents for the treatment of human diseases.
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5.5 Reference


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Chapter 6

A Newly Developed Glucose Responsive Microgel for
On-Site Tunable Self-Regulated Insulin Delivery

6.1 Introduction

Diabetes mellitus is a group of chronic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced\(^1\). This high sugar level in blood strongly impinges on a person’s quality of life and may lead to complications, such as blindness, nerve damage, foot or leg amputation, and stroke\(^2,3\). The advent of insulin revolutionized the treatment of diabetes mellitus and was one of the most outstanding achievements of twentieth century medicine. Exogenous insulin is administered subcutaneously to mimic the insulin secretion of the healthy pancreas. However, it is reported that only approximately 20% of insulin reaches the liver (primary site of action according to normal physiology) following the subcutaneous insulin injection\(^4\), resulting in hypoglycemia, insulin resistance, and other unwanted side effects of the excess insulin in the body\(^5,6\). Therefore, intelligent insulin administration to match physiological need of patients when necessary always remains a great challenge in numerous research efforts. While varies of
approaches can be envisioned to achieve this objective, considerable research has focused on the
development of self-regulated insulin delivery systems based on glucose-responsive polymers\textsuperscript{7–9}.

Three kinds of typical glucose-responsive materials have been used to develop insulin
delivery system. In early studies, glucose oxidase and concanavalin A were very popular due to
their versatility and high specificity. However, the reliance on protein-based components
presented severe limitations such as potential instability during use or sterilization\textsuperscript{10}. Conversely,
a synthetic ligand phenylboronic acids (PBA) and its derivatives were explored as substitutes for
natural receptors because of their great stability, longtime storability, and low cytotoxicity\textsuperscript{11, 12}. In aqueous solution, PBA is in equilibrium between undissociated neutral trigonal form (1) and a
dissociated anionic tetrahedral form (2) (Scheme 6–1). Both forms undergo reaction reversibly
with 1, 2-cis-diols such as glucose. While cyclic boronic esters between the neutral boronic acid
and a diol are generally considered hydrolytically unstable, the anionic form (2) is able to
reversibly bind with diols to form a 5- or 6-membered ring cyclic boronate ester (3), shifting the
equilibrium to the anionic forms (2 and 3)\textsuperscript{13}. The glucose-PBA complexation also changes the
polymer–solvent affinity. PBA is hydrophobic in the neutral state, which becomes more
hydrophilic upon complexation. The better polymer-solvent affinity and the increase in the
Donnan potential\textsuperscript{14} can both induce the volume phase transition (swelling) of the PBA-
containing hydrogels. Accordingly, the glucose-triggered insulin release from these systems lies
in one of the two different mechanisms: (1) altering the diffusion behavior of the preloaded
insulin molecules by glucose-responsive volume phase transition, (2) exchanging of the
preloaded insulin with glucose, or the synergistic effect from the combination of these two
mechanisms.
However, because of the high $pK_a$ of the PBA groups the working pH (≥ 8.5) of these insulin delivery systems is much higher than the physiological pH of 7.4. Two methods have been proposed to enhance the glucose sensitivity of the PBA moiety at physiological pH: (a) designing boronate analogues with lower $pK_a$ values; (b) introducing intramolecular $B^{δ−}−X^{δ+}$ bonds that confer a tetrahedral conformation at the boron centre through the neighboring effect of an ortho group. While sometimes the preparation protocols for the boronic acid derivatives with strongly electron-withdrawing substituents are rigorous and time-consuming, the intramolecular $B^{δ−}−X^{δ+}$ attraction approach is more appealing. We recently synthesized a new class of hybrid nanogels made of Ag nanoparticle cores covered by a glucose-responsive copolymer gel shell of poly(4-vinylphenylboronic acid-co-2-(dimethylamino) ethyl acrylate) [poly(VPBA-DMAEA)]. It was demonstrated that this Lewis base DMAEA with small degree of ionization can successfully enhance the binding affinity of PBA to glucose at physiological pH, thus the release of the encapsulated insulin can be realized at physiological pH. However, small amount of glucose can induce the swelling of the poly(VPBA-DMAEA) nanogels, resulting in the release of significant amount of insulin from the nanogels even at the low glucose concentrations below the normal blood glucose level (4–8 mM). The released insulin will decrease the glucose level in blood, thus may lead to serious hypoglycemia. Therefore, the ideal
insulin delivery carrier is supposed to initiate the release of insulin only after the glucose concentrations in blood are above the normal glucose level.

Herein, we design a novel class of biocompatible microgels copolymerized from VPBA, DMAEA, and oligo(ethylene glycol)methyl ether methacrylate(MEO₅MA) (Scheme 6–2) for on-site tunable glucose responsive insulin release. The resulted poly(VPBA-DMAEA-MEO₅MA) microgels upon a rational design of the polymer chains can undergo a volume phase transition in response to the change in glucose concentration with high sensitivity and selectivity over a clinically relevant range (0–30 mM) at the physiological pH and temperature. Copolymerization of MEO₅MA can not only increase the hydrophilicity and biocompatibility, but also shift the onset of the glucose-responsive volume phase transition of the microgels to desirable glucose level. The microgels contain both ethylene glycol and PBA moieties, thus the addition of glucose into the microgel solution can have two types of competitive interactions. One is the formation of hydrogen bonds between the ether oxygen of the ethylene glycol units and the hydroxyl groups of glucose molecules, which will not induce the microgels to swell. Another is the boronic acid-cis-diol complexation between the PBA groups and glucose molecules, which will induce the microgels to swell due to the production of negative charges. Because the formation of hydrogen bonds between glucose and ethylene glycol is much faster than the PBA-glucose complexation, only after the ether oxygen atoms of the polyMEO₅MA segments are saturated with glucose molecules, free glucose molecules will start to bind with PBA group to induce the swelling of microgels. Therefore, we can control the onset of glucose responsive volume phase transition of the microgels through the feeding ratios of comonomers of MEO₅MA to VPBA in synthesis. Such rationally designed biocompatible microgels that can intelligently dose insulin only after
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the glucose concentration above desirable level may hasten the development of more efficacious systems toward a self-regulated insulin delivery for good diabetic control.

We also synthesized glucose-imprinted microgels by polymerization of comonomers in the presence of the molecular target (glucose). It is expected that the collective weak interactions between the VPBA monomers and the glucose target molecules can form populations of complementary binding sites favorable for glucose bindings in the resulted polymer chain networks\textsuperscript{24, 25}, which should increase the sensitivity of glucose. Additionally, the embedding of Ag nanoparticles (NPs) to the glucose-imprinted microgels can successfully convert the biochemical signal (glucose level here) into optical signals, realizing a combination of optical detection of glucose and self-regulated insulin release. The insulin-loading capacity, sustained insulin release behavior, and cytotoxicity of the microgels were also evaluated.

Scheme 6–2 Schematic illustration of poly(VPBA-DMAEA-MEO\textsubscript{5}MA) microgels that can tune the onset for glucose-induced volume phase transition only after the glucose concentration beyond a certain level.
6.2 Experimental

6.2.1 Materials

D(+)-Glucose was purchased from ACROS, and all other chemicals were purchased from Aldrich. Oligo(ethylene glycol) methyl ether methacrylate (Mn = 300 g/mol, MEO₅MA) and 2-(Dimethylamino)ethyl acrylate (DMAEA) was purified with neutral Al₂O₃. The lyophilized fluorescein isothiocyanate-labeled insulin (FITC-insulin) from bovine pancreas (~5800 Da), 4-vinylphenylboronic acid (VPBA), N,N’-methylene bisacrylamide (MBAAm), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium dodecyl sulfate (SDS), dulbecco’s modified eagle medium (DMEM), and fetal bovine serum (FBS) were used as received without further purification. The water used in all experiments was of Millipore Milli-Q grade.

6.2.2 Synthesis of glucose responsive poly(VPBA-DMAEA-MEO₅MA) microgels

In a 250 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, VPBA (1.63 mmol) and 100 mL deionized water were heated to 70 °C. After VPBA was totally dissolved, DMAEA (0.329 mmol), MBAAm (0.002g), SDS (0.351 mmol), and MEO₅MA with designed concentrations were successively added to the solution under stirring. After the temperature was maintained at 70 °C and a continuous N₂ purge for 30 min, the polymerization was initiated by adding 1 mL of 0.105 M AAPH. The polymerization reaction was allowed to proceed for 5h. The dispersion was centrifuged at 6000 rpm (30 min, Thermo Electron Co. SORVALL® RC-6 PLUS super-speed centrifuge) with the supernatant discarded and the precipitate was redispersed in 100 mL deionized water. The resultant microgels were further purified by 3 days of dialysis (Spectra/Por molecular porous membrane tubing, cutoff 12000–14000 Da MWCO, the same as below) against very frequently changed water at room
temperature (~22 °C). The resultant microgels made with feeding of MEO₅MA = 0, 0.163, 0.326, 0.489, and 0.652 mmol were coded as VDM–0, VDM–1, VDM–2, VDM–3, and VDM–4, respectively, and the recipes for the synthesis of the poly(VPBA-DMAEA-MEO₅MA) microgels are listed in Table 6–1.

Table 6–1
Feeding compositions for synthesis of the poly(VPBA-DMAEA-MEO₅MA) microgels.

<table>
<thead>
<tr>
<th>Sample (mmol)</th>
<th>VPBA</th>
<th>DMAEA</th>
<th>MEO₅MA</th>
<th>Glucose</th>
<th>SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-imprinted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDM–0</td>
<td>1.63</td>
<td>0.329</td>
<td>–</td>
<td>–</td>
<td>0.351</td>
</tr>
<tr>
<td>VDM–1</td>
<td>1.63</td>
<td>0.329</td>
<td>0.163</td>
<td>–</td>
<td>0.351</td>
</tr>
<tr>
<td>VDM–2</td>
<td>1.63</td>
<td>0.329</td>
<td>0.326</td>
<td>–</td>
<td>0.351</td>
</tr>
<tr>
<td>VDM–3</td>
<td>1.63</td>
<td>0.329</td>
<td>0.489</td>
<td>–</td>
<td>0.351</td>
</tr>
<tr>
<td>VDM–4</td>
<td>1.63</td>
<td>0.329</td>
<td>0.652</td>
<td>–</td>
<td>0.351</td>
</tr>
<tr>
<td>Imprinted microgels</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVD–1</td>
<td>1.63</td>
<td>0.329</td>
<td>0.489</td>
<td>0.82</td>
<td>0.351</td>
</tr>
<tr>
<td>IVD–2</td>
<td>1.63</td>
<td>0.329</td>
<td>0.489</td>
<td>1.63</td>
<td>0.351</td>
</tr>
</tbody>
</table>

6.2.3 Synthesis of imprinted glucose responsive poly(VPBA-DMAEA-MEO₅MA) microgels

In a 250 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, VPBA (1.63 mmol) and 100 mL glucose aqueous solution (pH = 8.8) with a designed concentration were heated to 70 °C. After VPBA was completely dissolved, DMAEA (0.329 mmol), MBAAm (0.002g), SDS (0.0.351 mmol), and MEO₅MA (0.489 mmol) were successively added to the solution under stirring. After a continuous N₂ purge at 70 °C for 1 h, the polymerization was initiated by adding 1 mL of 0.105 M AAPH. The polymerization reaction was allowed to proceed for 5h. The dispersion was centrifuged at 6000 rpm (30 min, Thermo Electron Co. SORVALL® RC-6 PLUS super-speed centrifuge) with the supernatant discarded.
and the precipitate was redispersed in 100 mL deionized water. The resultant glucose-imprinted microgels were further purified by dialysis against very frequently changed water at room temperature (~22 °C) for at least two weeks. The resultant microgels with low and high glucose imprinting degrees were coded as IVD–1 and IVD–2, respectively (Table 6–1).

6. 2. 4 Insulin loading and release

FITC-insulin was loaded into the microgels by complexation method. A stock solution of FITC-insulin (1 mg/mL) was prepared in 0.005 M phosphate buffer solution (PBS) of pH = 7.4, and stored in the refrigerator (4 °C). The pH value of the microgel dispersion (5 mL) was adjusted to 9.0 by using diluted NaOH solution. This dispersion was stirred in an ice water bath for 30 min and then 1 mL of FITC-insulin solution was added dropwise to the vial. The immediate clouding phenomenon revealed the hydrogen bonding complexation of the insulin molecules (protein) with the ethylene glycol units and boronic acid groups in the microgels. After stirring overnight, the suspension was centrifuged at 6000 rpm for 30 min at 22 °C. To remove the free drug molecules, the precipitate was redispersed in 5 mL of PBS of pH = 7.4 and further purified by repeated centrifugation and washing. Finally, the precipitate of insulin-loaded microgels was redispersed in 1 mL of PBS of pH = 7.4. All the upper clear solutions were collected, and the concentration of free insulin was determined by fluorescence spectrometry at 518 nm upon excitation at 492 nm. The amount of loaded FITC-insulin in the microgels was calculated from the decrease in drug concentration. The loading content is expressed as the mass of loaded drug per unit weight of dried microgels.

The in vitro release test of FITC-insulin from the microgels was evaluated by the dialysis method. A dialysis bag filled with 1mL of purified insulin-loaded microgel dispersion was
immersed in 50 mL of 0.005 M buffer solutions of pH = 7.4 at various glucose concentrations. The released FITC-insulin outside of the dialysis bag was sampled at defined time intervals and assayed by fluorescence spectrometry at 518 nm upon excitation at 492 nm. Cumulative release is expressed as the total percentage of insulin released through the dialysis membrane overtime.

6.2.5 In vitro cytotoxicity

B16F10 cells (2000 cell/well) were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin in a 96-well plate, and exposed to microgels VDM−(1–4) respectively. To cover the high concentrations, the microgels were concentrated and adjusted to an appropriate concentration in DMEM right before feeding into the wells. The plate was incubated at 37 °C for 24 h. The medium was then aspirated, and these wells were washed using fresh serum-free DMEM. After that, 25 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) were added to the wells. After incubation for 2 h, the solution was aspirated and 100 µL of dimethyl sulfoxide was added to each well, and the plate was sealed and incubated for 30 min at 37 °C with gentle mixing. Three portions of the solution obtained from each well were transferred to three respective well of a 96-well plate. Cell viability was measured using a microplate reader at 570 nm. Positive controls contained no microgels, and negative controls contained MTT.
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6. 2. 6 Synthesis of hybrid microgels with Ag NP as core and the glucose-imprinted poly(VPBA-DMAEA-MEO₅MA) gel as shell

6. 2. 6. 1 Synthesis of Ag NPs

Citrate-stabilized Ag NPs were first prepared by dropwise addition of fresh NaBH₄ solution (10.6 mM, 2.5 mL) to an aqueous solution of AgNO₃ (0.1 mM, 200 mL) in the presence of sodium citrate (0.1 mM) under vigorous stirring. The resultant solution was stirred for 1 h and aged for 7 days at ambient conditions before use. The long aging time is necessary for completely degrading the reducing agent of NaBH₄. SDS stabilized Ag NPs were obtained by adding 0.053 g SDS into 100 mL of aqueous disperson of citrate-stabilized Ag NPs in a 250 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, and then aging the mixture overnight (~10 h).

6. 2. 6. 2 Synthesis of Ag@glucose-imprinted poly(VPBA-DMAEA-MEO₅MA) hybrid microgels

In a 250 mL round-bottom flask equipped with a stirrer, a N₂ gas inlet, and a condenser, 100 mL of as-prepared aqueous solution (pH = 8.8) of SDS stabilized Ag NPs was heated to 30 °C, followed by addition of VPBA(0.4075 mmol), DMAEA(0.0825 mmol), MBAAm (0.0005 g), SDS (0.0878 mmol), MEO₅MA (0.1223 mmol), and glucose(0.4075 mmol) under stirring (one quarter recipe of IVD–2, Table 6–1). After a continuous N₂ purge for 1 h, the temperature was raised to 70 °C and the polymerization was initiated by adding 1 mL of 0.105 M AAPH. The polymerization reaction was allowed to proceed for 5 h. The dispersion was centrifuged at 6000 rpm (30 min, Thermo Electron Co. SORVALL® RC-6 PLUS super-speed centrifuge) with the supernatant discarded and the precipitate was redispersed in 100 mL deionized water. The
resultant hybrid microgels were further purified by dialysis against very frequently changed water at room temperature (~22 °C) for at least two weeks. The resultant hybrid microgels were coded as Ag@IVD−2.

6.2.7 Characterization

The morphology of the microgels was characterized with transmission electron microscopy (TEM). The TEM images were taken on a FEI TECNAI transmission electron microscope at an accelerating voltage of 120 kV. Approximately 10 µL of diluted microgel suspension was dropped on a Formvar covered copper grid (300 meshes) and then air-dried at room temperature for the TEM measurements. The UV–vis absorption spectra were obtained on a Thermo Electron Co. Helios UV–vis spectrometer. The PL spectra were respectively obtained on a JOBIN YVON Co. FluoroMax®-3 Spectro fluorometer equipped with a Hamamatsu R928P photomultiplier tube, calibrated photodiode for excitation reference correction from 200 to 980 nm, and an integration time of 1 s. The pH values were obtained on a METTLER TOLEDO SevenEasy pH meter.

Dynamic light scattering (DLS) was performed on a standard laser light scattering spectrometer (BI-200SM) equipped with a BI-9000 AT digital time correlator (Brookhaven Instruments, Inc.). A Nd:YAG laser (150 mW, 532 nm) was used as the light source. All microgel solutions were passed through Millipore Millex-HV filter with a pore size of 0.80 µm to remove dust before the DLS measurements. In DLS, the Laplace inversion of each measured intensity-intensity time correlated function can result in a characteristic line width distribution $G(\Gamma)$. For a purely dissipative relaxation, $\Gamma$ is related to the translational diffusion coefficient $D$ by $(\Gamma/q^2)_{c\rightarrow0,q\rightarrow0} = D$, where $q = (4\pi n/\lambda)\sin(\theta/2)$ with $n$, $\lambda$, and $\theta$ being the solvent refractive
index, the wavelength of the incident light in vacuo, and the scattering angle, respectively. \( G(D) \) can be further converted to a hydrodynamic radius \( (R_h) \) distribution by using the Stokes-Einstein equation, 
\[
R_h = \left( \frac{k_B T}{6 \pi \eta} \right) D^{-1},
\]
where \( k_B, T, \) and \( \eta \) are the Boltzmann constant, the absolute temperature, and the solvent viscosity, respectively.

6.3 Results and Discussion

6.3.1 Synthesis of glucose responsive poly(VPBA-DMAEA-MEO5MA) microgels

The glucose responsive poly(VPBA-DMAEA-MEO5MA) microgels were synthesized from the free radical precipitation copolymerization of VPBA, DMAEA, and MEO5MA using MBAAm as a crosslinker (Table 6–1). Figure 6–1 shows the typical TEM image of the poly(VPBA-DMAEA-MEO5MA) microgels VDM–3. The resultant particles have a spherical morphology. The DLS results indicate that the microgels synthesized with only VPBA and DMAEA but no MEO5MA (as a control), coded as VDM-0, have the smallest size with \( R_h = 83 \) nm. After being copolymerized with MEO5MA, the microgel particles become larger. The microgels VDM–1, VDM–2, VDM–3, and VDM–4 with an increased MEO5MA feeding of 7.68 mol\%, 14.27 mol\%, 19.98 mol\%, and 24.97 mol\% have an increased \( R_h \) of 106, 118, 142 and 148 nm, respectively, indicating that the increase in the feeding amount of comonomer of MEO5MA can significantly increase the size of the microgels. The size increase of the microgels at higher feedings of MEO5MA should be attributed to the decrease of the feeding ratios of surfactant SDS to the total comonomers. While we fixed the same feeding amount of comonomers of VPBA and DMAEA and the dispersing agent of SDS, we gradually increased the feeding of comonomer of MEO5MA. Relatively, less SDS molecules are available for the comonomers, resulting in less nuclei particles being formed in the precipitation polymerization.
process and larger microgel particle size. The controllable size of the microgels is very important to their fate in the bloodstream. Recognition by the reticuloendothelia system (RES) is known to be the principal reason for the removal of many colloidal drug carriers from the blood compartment\textsuperscript{26}. The sub-200 nm size is desirable for colloidal particles to extend their blood circulation time.

![Figure 6-1](image)

**Figure 6-1** Typical TEM image of the poly(VPBA-DMAEA-MEO\textsubscript{3}MA) microgels (VDM–3).

The previous method to fabricate glucose-responsive polymeric microgels involves the subsequent functionalization of carboxylated microgels with aminophenylboronic acid (APBA) through the carbodiimide-catalyzed coupling reaction\textsuperscript{27–37}. Our group have previously employed poly(VPBA-DMAEA) based polymers to fabricate polymeric micro/nanogel with high glucose sensitivity and selectivity at physiological pH and temperature because the small degree of ionization of the polyDMAEA segments with positive charge can enhance the binding affinity of PBA to glucose at physiological pH\textsuperscript{21}. This result has been further confirmed in this work. Figure
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6–2 shows the pH-induced volume phase transitions of the microgels in terms of the change of hydrodynamic radius ($R_h$) measured at 37 °C. The pH-dependent volume phase transition has been observed due to the presence of pH-sensitive functional groups from the DMAEA ($pK_b \approx 7.5$) and VPBA ($pK_a \approx 8.9$) components. Both the protonation of the amino groups in the polyDMAEA segments and the donation of proton from the boronic acid groups in the polyVPBA segments can increase the ionization degree of the copolymer chains, thus induce the swelling of the microgels with larger $R_h$. The amino groups in the polyDMAEA segments are slightly ionized at the physiological pH. The further decrease in pH from the physiological pH can increase the ionization degree of polyDMAEA segments with positive charges, thus increase the size of microgels. The copolymer microgels demonstrated the smallest size at pH = 7.7, where both polyDMAEA ($pK_b \approx 7.5$) and polyVPBA ($pK_a \approx 8.9$) segments have a minimum degree of ionization. The continuous increase in pH from pH = 7.7 will increase the ionization degree of polyVPBA segments with negative charges, thus can also induce the swelling of microgels and increase their size.

![Figure 6–2 pH-dependent average $R_h$ values of the microgels VDM-0 (■), VDM-1 (●), and VDM-3 (▲). All measurement were made at 37 °C and a scattering angle $\theta = 45^\circ$.](image-url)
6.3.2 Glucose-induced volume phase transition

It is known that the PBA group is in equilibrium between the undissociated (trigonal, uncharged) and the dissociated (tetrahedral, charged) forms in aqueous solution\(^{38}\). Both forms react reversibly with 1,2-cis-diols such as glucose, but the binding of glucose induces the thermodynamically more favorable charged form. The presence of glucose can move the dissociation equilibrium of PBA to the right and further decrease its \(pK_a\), so if a strong intramolecular \(B^{\delta-}\cdots X^{\delta+}\) bond can be generated, then such boronic acids would bind glucose at physiological pH. In our poly(VPBA-DMAEA-MEO\(_5\)MA) microgels, the small degree of ionization of the polyDMAEA segments can also enhance the binding affinity of PBA to glucose at physiological pH range through the intramolecular \(B^{\delta-}\cdots X^{\delta+}\) bonding between the boronate ester and the adjacent slightly protonated dimethyl amino groups\(^{19,39-41}\). Figure 6–3 shows the glucose induced swelling curves of the copolymer microgels, in terms of the \(R_{h,[\text{glucose}]}/R_{h,[\text{glucose}]=0}\) measured in a 5.0 mM PBS of pH = 7.4 at 37 °C and different glucose concentrations. The binding of glucose with the microgels produces negatively charged boronates and builds up a Donnan potential for the microgels to swell\(^{37}\). The polyMEO\(_5\)MA segments play two very important roles for the copolymer microgels. First, polyMEO\(_5\)MA segments can improve the hydrophilicity and biocompatibility of the microgels. According to our previous work, the polyMEO\(_5\)MA microgels should have a LCST above 50 °C\(^{42}\). Therefore, the local polyMEO\(_5\)MA gel domains in the microgel particles are in a swollen state at our study temperature of 37 °C, which should lead the microgels more stable in aqueous buffer solution. Second, the microgels with different comonomer MEO\(_5\)MA/VPBA feeding molar ratios exhibited different glucose responsive swelling behavior. As shown in Figure 6–3, both VDM–0 without any addition of MEO\(_5\)MA monomer and VDM–1 with a low feeding of MEO\(_5\)MA
monomer exhibited a similar sharp increase in particle size even at a very low glucose concentration. The glucose-induced maximum swelling ratio of VDM–1 was slightly lower than that of VDM–0. The continuous gradual increase in the feeding molar ratios of MEO5MA/VPBA comonomers in the synthesis of VDM–2, VDM–3, and VDM–4 can further decrease the glucose-induced maximum swelling ratio of microgels, which could be attributed to the relatively lower content of glucose-sensitive PBA components in the microgels because we fixed the same feeding of VPBA and DMAEA monomers, but increased the feeding of MEO5MA monomer. More importantly, one can clearly find that with the increase in the feeding ratio of MEO5MA/VPBA comonomers, the onset of the glucose responsive volume phase transitions of the resulted microgels gradually shifts to a higher glucose concentration. The higher the feeding ratio of MEO5MA/VPBA comonomers, the higher the glucose concentration required to initiate the glucose-responsive swelling of the microgels. For example, VDM–2, VDM–3, and VDM–4 microgels synthesized with the gradually increased content of MEO5MA comonomers demonstrated an increased initial glucose concentration of 2, 4, and 5 mM, respectively, to induce the glucose-responsive volume phase transitions of the microgels. These results should be attributed to the prior hydrogen bonding between the glucose and the polyMEO5MA segments in the microgels. Kinetic studies show that the formation and dissociation of intermolecular hydrogen bond in liquid solutions is ultrafast (e.g., on a picoseconds time scale)\textsuperscript{43}. On the other hand, the kinetics of the glucose-induced swelling of the PBA-containing microgels is on a time scale from seconds to ~100 seconds, depending on the glucose concentrations, due to the slow complexation reaction between the PBA groups and glucose molecules\textsuperscript{44, 45}. Thus, when the copolymer microgels containing both polypolyMEO5MA and polyVPBA segments were added into glucose solution, the glucose molecules will firstly bind to the polyMEO5MA segments via
hydrogen bonding. Only after the ether oxygens of the polyMEO5MA segments were saturated by glucose molecules, the boronic acid groups in the polyVPBA segments can access the remained glucose molecules to form negatively charged complexes and thus induce the swelling of microgels. Our results indicate that the content of the polyMEO5MA segments in the PBA-containing glucose-responsive microgels can control the onset of glucose responsive volume phase transition, which is very important to initiate and regulate the insulin release when these microgels are used as insulin delivery carriers.

Figure 6–3 Swelling ratio of the microgels VDM–0 ( ■), VDM–1 ( ●), VDM–2 ( ▲), VDM–3 ( ▼), and VDM–4 ( ♦) as a function of glucose concentration in PBS of pH = 7.4. All measurements were made at 37 °C and a scattering angle θ= 45°.

6.3.3 Insulin loading and in vitro glucose-regulated insulin release from the microgels

Having demonstrated the glucose-responsive volume phase transitions of the copolymer microgels, we further chose VDM–1, VDM–3 and VDM–4 as model drug carriers for loading
and delivery of FITC-insulin. The porous network structure of the microgels, together with the hydrogen bonding interactions between the insulin molecules and microgel network chains (containing ethylene glycol and boronic acid units), is particularly well suited to trap insulin, resulting in a high drug loading capacity. The yields of FITC-insulin loaded into the microgels were determined to be 33.3, 35.9, and 37.0 wt% (expressed as the mass of loaded insulin per unit weight of dried microgels) for VDM−1, VDM−3, and VDM−4, respectively. Considering the approximate concentrations of the microgel dispersions, these insulin loading capabilities are equivalent to the free insulin concentrations of 159.6, 163.2, and 160.0 mg/L, respectively. It should be noted that the microgels can be concentrated and adjusted to an appropriate concentration to cover an even higher insulin dose. Figure 6–4 shows the in vitro insulin releasing profiles from the microgel measured by the dialysis method for 48 hours in pH = 7.4 PBS at 37 °C. A blank release experiment of free FITC-insulin (~5800 Da) solution with an equivalent amount of drug to that trapped in VDM−1 was also performed, showing that the dialysis membrane (cutoff 12000 – 14000 Da) played a negligible role in the release kinetics. The much slower insulin release from the microgels compared to that from the free insulin solution indicates a sustained release process from the microgels. The release can be further regulated by varying the glucose concentration in the releasing medium because of the glucose responsive volume phase transitions of the PBA-containing microgels. The higher the glucose concentration in the medium, the more the insulin being released from the microgels, which is a basic requirement for self-regulated insulin delivery. For example, only 8.8% of insulin was released from VDM-1 after 48 h in PBS buffer solution without glucose. When the concentration of glucose was increased to 3, 6, 8 and 15 mM, the percentage of insulin released from VDM-1 reached to 49.6, 67.4, 75.3 and 89.2 %, respectively, during the same time period of 48 h. This
glucose dependent insulin release behavior should be attributed to the binding of glucose with the PBA groups in the copolymer microgels, producing negatively charged boronates and resulting in the microgel network to swell. The enlarged mesh size of the microgels is easier for the insulin molecules trapped in the interior of gel networks to diffuse out. Additionally, the copolymer microgels containing different amount of polyMEO₃MA segments played different release profiles. The insulin can be significantly released from the microgels containing low polyMEO₃MA segments even at low glucose concentrations of 3–6 mM, which is not desirable because the release of insulin at these low or normal glucose levels could cause hypoglycemia. In contrast, the increase in the content of polyMEO₃MA segments in the copolymer microgels can solve this critical problem. For example, at a normal glucose level of 6 mM, 67.4, 29.8, and 19.9 % of insulin can be released after 48 h from the VDM−1, VDM−3, and VDM−4 microgels, respectively. At abnormally high glucose level (e.g., 15 mM), all the three microgels can release out about 85% of the loaded insulin after 48 h. The insulin release profiles from microgels of VDM−3 and VDM−4 containing high polyMEO₃MA segments are more desirable for self-regulated insulin delivery to match the diabetic patients’ need, because ideal insulin delivery carriers should keep the loaded insulin minimally released at normal glucose levels but release rapidly upon reaching hyperglycemia to control glucose levels. First, these microgels containing high polyMEO₃MA segments only released minimum amount of insulin when the glucose concentration is below or in the normal level (4–8 mM)²²,²³, which could significantly reduce the potential hypoglycemia problem. Second, these microgels could start to deliver the insulin in less than 30 min when the glucose level is above the normal range. The sustained release over 2 days may also meet the basal need. Although the current results are obtained from in vitro
studies, these insulin releasing characteristics from the microgels containing high polyMEO_5MA segments clearly confers the advantages for the self-regulated insulin delivery systems.

**Figure 6-4** Releasing profiles of FITC-insulin from VDM-1 (A), VDM-3 (B), and VDM-4 (C), in the presence of 0 (■), 3(●), 6(▲), 8(▼), and 15(♦) mM glucose in PBS of 7.4. In the blank release (◊), the release experiment of the FITC-insulin solution with an equivalent amount of drug to that trapped in VDM-1 was performed in PBS of 7.4. All experiments were carried out at 37 °C.
6.3.4 *In vitro* cytotoxicity

![Graph showing cytotoxicity of microgels against B16F10 cells.]

**Figure 6–5** *In vitro* cytotoxicity of the microgels against B16F10 cells.

Although these microgels present immense opportunities on smart insulin release, it is known that the cytotoxicity of the drug carriers is extremely important for their future application. Cell viability was quantified by an MTT assay using B16F10 cells. MTT assay was first developed by Moamann in 1983.\(^{46}\) A mitochondrial dehydrogenation enzyme in viable cell can cleave the tetrazolim rings of the MTT, forming formazane crystals with a dark blue color. Therefore, the number of surviving cells is directly proportional to the level of the resulted formazane. Figure 6–5 shows the cell viability upon treatment with the microgel VDM–1, VDM–2, VDM–3, and VDM–4, respectively. It is clear that our poly(VPBA-DMAEA-MEO\(_5\)MA) copolymer microgels were non- or low-cytotoxic to B16F10 cells after incubation for 24 h at concentration of up to 208 µg/mL. The high contents of polyMEO\(_5\)MA segments in the VDM–3 and VDM–4 microgels also improved the cell viability due to the biocompatibility of the polyMEO\(_5\)MA components.
6.3.5 Glucose-induced volume phase transition of imprinted microgels

The imprinted poly(VPBA-DMAEA-MEO$_5$MA) microgels were prepared by one-pot method via free radical precipitation polymerization with the same recipe of VDM–3 (Table 6–1) but in presence of glucose, MBAAm, and SDS at 70 °C and pH = 8.8, as illustrated in Scheme 6–3. Previously, Willner’s group successfully imprinted glucose recognition sites by electro polymerization of a bis-aniline-crosslinked Au nanoparticles composite containing mercaptophenyl boronic acid on an Au electrode$^{47}$. Our group has exploited an alternative method by using glucose to complex the charged PBA groups of the comonomer VPBA at pH ≈ $pK_a$ (= 8.8) prior to polymerization$^{47}$. It is expected that when the collective weak interactions between the VPBA monomers and the target glucose molecules can form populations of complementary binding sites in the resulted polymer chain network$^{24, 25}$.

![Scheme 6–3](image)

Scheme 6–3 Schematic illustration of the approach to the molecularly imprinted poly(VPBA-DMAEA-MEO$_5$MA) microgels with specific glucose-binding sites for highly sensitive and selective glucose detection.
The swelling process of the glucose-imprinted poly(VPBA-DMAEA-MEO$_5$MA) microgel can also occur at physiological pH. Figure 6–6 shows the glucose-induced swelling curves in terms of the $R_h$ values as a function of glucose concentration of the non-imprinted microgel of VDM–3 and the corresponding imprinted microgels of IVD–1 and IVD–2, measured in a 5 mM PBS of pH = 7.4 at 37 °C. First, prepared from the same feeding ratio of all comonomers, the three copolymer microgels of VDM–3, IVD–1 and IVD–2 with the same high content of poly MEO$_5$MA segments exhibited the same onset of the glucose-induced volume phase transitions. The microgels swell very little when the glucose concentration was below ~3 mM. Significant glucose-induced increases in the swelling ratios were only clearly observed after the glucose concentration was above ~4 mM. As we discussed above, the glucose-ethylene glycol hydrogen bonding interaction and the glucose-PBA binding complexation are competitive processes. Glucose molecules would start to bind with PBA moieties to induce the swelling of microgels only after the ether oxygens of the polyMEO$_5$MA segments were saturated with glucose molecules. Second, the three microgels of VDM–3, IVD–1, and IVD–2 with a gradually increased glucose imprinting degree with [glucose]/[VPBA] ratio of 0, 1:2, and 1:1 in the synthesis exhibited an increased swelling ratio ($R_h$,$20mM$/$R_h$,$0mM$) of 1.61, 1.66, and 1.69, respectively. This result can be explained that the microgels with a high glucose imprinting degree can create and rigidly retain more binding sites complementary to the shape of the target glucose molecule in the crosslinked polymer network, thus possessing a higher affinity to glucose and producing more negatively charged glucose-bound boronate sites on the gel network chains.
6.3.6 FITC-insulin loading and *in vitro* glucose regulated insulin release from the imprinted microgels

The glucose-imprinted poly(VPBA-DMAEA-MEO<sub>5</sub>MA) microgels could also serve as excellent drug carriers benefited from the stable porous network structure under typical administration conditions and the hydrogen bonding complexations of the insulin molecules with the copolymer network chains. A drug loading capacity of 36.5 wt% and 36.7 wt% (expressed as the mass of loaded drug per unit weight of dried microgels) was determined for IVD–1 and IVD–2, respectively. As expected, the glucose imprinting degree of the microgels will not affect the drug loading capacity because all the three microgels of VDM–3, IVD–1, and IVD–2 were
synthesized with the same feeding of comonomers and crosslinkers, but in the presence of different amount of glucose.

The glucose-induced volume phase transitions of the imprinted microgels can also be used to regulate the release of the pre-loaded insulin. As shown in Figure 6–6, we have demonstrated that the increase in the glucose imprinting degree of microgels can slightly increase the glucose-induced swelling ratio of the microgels due to the improved glucose affinity of the imprinted PBA sites. To examine whether the small increase in the glucose-sensitive swelling ratio will affect the insulin releasing behavior from the microgels, the *in vitro* insulin release from the imprinted microgels of IVD–1 and IVD–2 was determined in buffer solutions of pH = 7.4 at 37 °C but different glucose concentrations (Figure 6–7). Similar to the insulin releasing behavior from the non-imprinted microgels, the glucose imprinted microgels also demonstrated a sustained release of the loaded insulin, which can be regulated by varying the glucose concentration in the surrounding medium. Because of the same high content of polyMEO₃MA segments in the microgels of VDM–3, IVD–1, and IVD–2, all these three microgels with different imprinting degree demonstrate a minimum release of insulin when glucose concentration was below 3 mM (Figure 6–4B for VDM–3, Figure 6–7 for IVD–1 and IVD–2). When glucose concentration was increased to 8 and 15 mM, the releasing percentage of insulin can reach to about 56 and 87%, respectively, after 48 h. These results demonstrate a great promise for the microgels to serve as a smart insulin delivery carrier. Similar to the effects on the glucose-induced volume phase transition, the gradual increase in the glucose imprinting degree of the microgels also leads to a slight increase in the insulin release capacity. For example, at glucose concentration of 15 mM, the releasing percentage of insulin of VDM–3, IVD–1, and IVD–2 was 86.8, 87.5, and 89.0%, respectively. This result should be related to the slightly
increased glucose-induced swelling degree of the imprinted microgels. The higher the glucose imprinting degree, the higher the glucose affinity of the microgels, which leads to a larger swelling degree and correspondingly a higher insulin release rate.

**Figure 6–7** Releasing profiles of FITC-insulin from IVD-1 (A) and IVD-2 (B), in the presence of 0 (■), 3(●), 6(▲), 8(▼), and 15(♦) mM glucose in PBS of 7.4. All experiments were carried out at 37 °C.
6.3.7 Optical glucose detection of the core-shell hybrid microgels with Ag NP as core and glucose-imprinted poly(VPBA-DMAEA-MEO₅MA) gel layer as shell

![Graph showing UV–vis absorption spectra](image)

**Figure 6–8** Typical UV–vis absorption spectra of the Ag@IVD-2 hybrid microgels. As a comparison, the absorption spectrum of the free Ag NPs is also presented.

Considering the glucose imprinting can create and rigidly retain more binding sites complementary to the shape of the target glucose molecule in the crosslinked polymer network, we expect that glucose imprinted microgels should demonstrate high sensitivity and selectivity for optical glucose detection if an optical code can be installed into the microgels. Here, we synthesized a core-shell hybrid microgel using the fluorescent Ag NPs as the core template and the imprinted microgel layer (same composition as the IVD-2) as shell in order to convert the glucose level change (biochemical signal) into the fluorescence change of Ag NPs (optical signals). After the coating of the gel layer, the absorption peak of the Ag NPs red-shifted by ∼13 nm (Figure 6–8). This red-shift is related to a change in the local dielectric constant around the Ag NPs resulted from the coating of the poly(VPBA-DMAEA-MEO₅MA) gel layer onto the Ag
NPs. As shown in Figure 6–9, the resultant core-shell particles have a spherical morphology. The markedly high electron density of Ag enables direct visualization of the Ag NP core within the poly(VPBA-DMAEA-MEO₃MA) microgels.

**Figure 6–9** TEM image of the core-shell hybrid microgels Ag@IVD-2 hybrid microgels with Ag NP as core and glucose-imprinted poly(VPBA-DMAEA-MEO₃MA) gel layer as shell.

When the Ag@IVD-2 hybrid microgels were dispersed in PBS at pH = 7.4, robust photoluminescence (PL) can be detected, because the size of Ag NPs is far smaller than the electron mean free path length (∼ 50 nm)⁴⁸,⁴⁹. The reversible glucose-responsive volume phase transitions (swelling-shrinking transitions) of the IVD–2 gel shell can modify the physicochemical environment and surface tension of the Ag core NPs, thus induce the photoluminescence (PL) change of Ag NPs. Figure 6–11 shows the PL spectra of the Ag@IVD-2 hybrid microgels dispersed in PBS buffer of pH = 7.4 but with different glucose concentrations. The Ag@IVD-2 hybrid microgels demonstrate a strong peak at about 588 nm, which should be attributed to the PL produced from the core Ag NPs encapsulated in the IVD–2 microgels. It is
clear that the PL intensity of the hybrid microgels was gradually quenched when the microgels gradually swelled up at the elevated glucose concentration. One reason for the PL property change of Ag NP cores in the hybrid microgels is associated with the variation of the Rayleigh scattering through the local refractive index change of the medium surrounding the Ag NPs, deriving from the glucose-induced volume phase transition of the microgel\textsuperscript{50}. The non-radiative energy loss paths, which are highly dependent on the nature of the environment around the metal particles, possibly provide a second scenario for the fluorescence change\textsuperscript{51}. The crosslinked copolymer chains tend to expand as the glucose concentration increases. On the other hand, the Ag core NPs offer the restoring force to hinder the network expansion, introducing an elastic tension in the bond at the Ag-polymer interface that would induce strain at the relaxed Ag NP surface\textsuperscript{52}, creating interfacial states that quench the PL.
**Figure 6–10** PL spectra of the Ag NPs, Ag@IVD-2 hybrid microgels, and IVD–2 microgels, respectively.

**Figure 6–11** Typical evolution of the PL spectra of the Ag@IVD-2 hybrid microgels in response to the increase in glucose concentration change. All measurements were made in PBS of pH = 7.4 and 37 °C. Excitation wavelength = 492 nm.
Figure 6−12 summarizes the quenched PL intensity as a function of glucose concentration. Interestingly, this plot of the glucose responsive PL property change demonstrates a similar trend to the glucose responsive swelling curve (Figure 6−6). When the concentration of glucose was increased from 0 to 3 mM, the PL signal at 588 nm of the hybrid microgels only quenched by ~5%. As the glucose concentration was above 4 mM, the increase in glucose concentration can induce a dramatic PL quenching until the glucose concentration reaches about 16 mM where the PL quenching leveled off, corresponding to the same glucose level at which the microgel network chains stretched to nearly a maximum (Figure 6−6). Another important finding is that the glucose molecular imprinting can dramatically improve the sensitivity of the hybrid microgels for glucose detection. As shown in Figure 6-6, the imprinted hybrid microgels of Ag@IVD−2 demonstrated a slightly higher swelling ratio of \( \frac{R_{h,20mM}}{R_{h,0mM}} = 1.69 \) compared to the nonimprinted hybrid microgels of Ag@VDM−3 with \( \frac{R_{h,20mM}}{R_{h,0mM}} = 1.61 \). The PL response to the glucose concentration change in terms of a quenched PL intensity shows a significantly different value of \( \frac{(I_0−I_{20mM})}{I_0} = 0.86 \) and 0.50, respectively for the Ag@IVD−2 and Ag@VDM−3 hybrid microgels. This result indicates that the slightly larger volume response of the imprinted Ag@IVD−2 to the same glucose concentration (e.g. 20 mM) has been dramatically amplified to as large as ~1.64 times in the PL response, demonstrating the enhancement effect of the molecular imprinting on the glucose-binding ability. Additional experiment of glucose-responsive PL decay of the imprinted imprinted Ag@IVD−3 (\( n_{VPBA} : n_{glucose} = 1:1.5 \) presented in polymerization) has also performed. The slightly different value of \( \frac{(I_0−I_{20mM})}{I_0} = 0.97 \), comparing 0.86 of Ag@IVD−2, indicating glucose bonded most of PBA group during the polymerization. The glucose-binding ability of imprinted microgels would not change too much if more glucose presented in polymerization.
Figure 6–12 Glucose-responsive PL decay \((I_0 - I)/I_0\) of the imprinted (Ag@IVD–2, ●) and non-imprinted (Ag@VDM–3, ■) hybrid microgels, respectively. Glucose-responsive PL decay of the imprinted imprinted Ag@IVD–3 (▲, \(n_{VPBA} : n_{glucose} = 1:1.5\) presented in polymerization) has also performed. \(I_0\) and \(I\) are the PL intensity at 588 nm in the absence and presence of glucose at different level. All measurements were made in PBS of \(pH = 7.4\) and 37 °C. Excitation wavelength = 492 nm.

6.4 Conclusion

Well-defined glucose-sensitive poly(VPBA-DMAEA-MEO₅MA) copolymer microgels could be successfully synthesized via a simple precipitation polymerization method. The presence of polyMEO₅MA segments could retard the poly(VPBA-DMAEA-MEO₅MA) copolymer microgel network from swelling in response to the addition of glucose. The reason is that the formation of hydrogen bonding between the glucose molecules and the ether oxygens of the polyMEO₅MA segments, which causes no swelling of microgels, is much faster than the glucose binding complexation to the PBA groups, which leads to a swelling of microgels. The
higher the content of the polyMEO\textsubscript{5}MA segments in the copolymer microgels, the higher the glucose concentration required to initiate the glucose sensitive volume phase transition. Thus, the onset point of glucose sensitivity of the copolymer microgels could be tuned through the adjustment of the feeding amount of MEO\textsubscript{5}MA comonomer in the preparation of microgels. These newly designed biocompatible glucose sensitive microgels are promising for smart insulin delivery carriers, which can release minimum amount of insulin when the glucose level is below or in the normal range, but start to release insulin rapidly when the glucose level is above the normal range. The higher the glucose level, the faster the insulin release from the microgels. Compared to the non-imprinted copolymer microgels, the glucose imprinting of the microgels can create and rigidly retain more binding sites complementary to the shape of the target glucose molecule in the crosslinked polymer network, thus improve the sensitivity and selectivity of the microgels in response to the glucose level change. Additionally, the introduction of fluorescent Ag NPs to the microgels can realize the integration of optical glucose detection and self-regulated insulin delivery into a single nano-object.
6.5 Reference


Chapter 6


Chapter 6


Chapter 7

Summary

The overall goal of the work in this dissertation was to investigate the design, properties and applications of multi-responsive microgels, especially, their role act as cell scaffolds, particular carriers for controlled and targeted drug delivery system and response to different environmental stimuli.

A cell scaffold could be realized by colloidal supra-structures, which constructed by the thermo-driven gelation of the colloidal dispersion of poly(NIPAM-co-AAm) microgels. Different from the previous examples in which the VPTT of the temperature-responsive microgels are much below 37 °C, making them fully collapsed and possibly hard-sphere-like under the physiological conditions, the presented poly(NIPAM-co-AAm) microgels exhibit a reversible and continuous volume transition in water with VPTT ≈ 35 °C and remain partially swollen and soft under physiological conditions. The constructed colloidal supra-structure can be regarded as a model system for a new class of cell scaffolds. More importantly, the size of the microgel particles can affect the sol-to-gel phase transition of the microgel dispersions, alter the syneresis degree of the constructed colloidal supra-structures, and tailor the cytocompatibility when the colloidal supra-structures were used for cell scaffolds.
Microgel can also be applied as a drug delivery carrier. In Chapter 4, well-defined thermo-responsive PVAS@PEG core-shell microgels with the PVAS microgels as hydrophobic core and the nonlinear PEG gel as a hydrophilic shell could be successfully synthesized via precipitation polymerization. The rationally designed PVAS core chain networks can effectively store the hydrophobic curcumin drug molecules via hydrophobic interactions, thus provide high drug loading capacity. The nonlinear PEG gel shell composed of the copolymer P(MEO_{2}MA-co-MEO_{5}MA) with the comonomer ratio of MEO_{2}MA:MEO_{5}MA = 1:2 could not only increase the stability of the core-shell microgels dispersion in aqueous media, but also prevent the PVAS hydrophobic core network from collapsing. This designed structure of hydrophobic core with a large mesh size in the core-shell microgels can load hydrophobic drug molecules much more effectively, which indicates that both the drug-core hydrophobic interactions and the mesh size of core networks are important to determine the drug loading capacity. The thermo-sensitive nonlinear PEG gel shell of the microgels could trigger the drug release by local temperature change, offering sustained drug release profiles.

A magnetic Fe_{3}O_{4} NPs has been involved in microgel in Chapter 5. The magnetic hybrid nanogels appear to be quite suitable for being a drug targeted carrier, due to their capability of delivering pharmaceuticals to a specific site of the body by means of a gradient magnetic field. The core-shell structure nanogel composed of the magnetic Fe_{3}O_{4} NPs core and poly(4-allylanisole)-PEG double-layer gel shell could be successfully synthesized via precipitation polymerization. 4-allylanisole, a natural organic compound produced by many conifers and herbs, is general consider as a biocompatible polymer. It is usually used in the preparation of fragrances and a flavoring additive of bakery products and beverages. Similar like PVAS in Chapter 4, the hydrophobic aromatic groups of rational designed poly(4-allylanisole) inner shell
gel can form strong hydrophobic associations with the aromatic phenols in curcumin molecules to form intermolecular complexes, resulting in relative high drug loading capacity.

In Chapter 6, a class of well-defined glucose-sensitive microgels polymerization from VPBA, DMAEA, and MEO₅MA is synthesized as an insulin drug release carrier. The presence of MEO₅MA monomer could retard the glucose-sensitive network from swelling because the rapid hydrogen bonding between the glucose molecules and the ether oxygens of the MEO₅MA is prior to the glucose binding to the PBA groups. The onset point of glucose sensitivity of the microgels could be controlled through the adjustment of the feeding amount of MEO₅MA monomer. Glucose-imprinted microgels that polymerized in the presence of glucose can increases sensitivity of glucose. The introducing of Ag NPs to the microgel realized the integration of glucose detection and self-regulated insulin delivery into a single nano-object. These newly designed biocompatible glucose sensitive microgels are promising for insulin delivery carriers, which can detect the glucose level at the same time as well as regulate the insulin release from the microgel only after the glucose level is above critical zone in diabetes management.