Development and characterization of chitosan-based novel gene carrier systems

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Development and Characterization of Chitosan-Based Novel Gene Carrier Systems

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Abstract

Gene delivery research has grown very rapidly due to its huge potential as a therapeutic strategy for clinical applications to treat many inheritable or acquired diseases by replacing defective genes, substituting missing genes, or silencing unwanted gene expression. However, the lack of effective carriers is a major barrier for development of human gene therapy. Cationic polymers have been known as potential candidates for making non-viral gene vectors (carriers). Among them, chitosan (CS) has been considered to a good gene carrier candidate, because CS is known as biocompatible, biodegradable, low toxicity, and high cationic potential.

The shorter length and linearity of siRNA is the prime feature motivating researchers to chemically modify a conventional CS based DNA delivery system. The interaction between CS and siRNA by simple mixing is apparently weak, thus CS and siRNA are easily dissociated from each other. Grafting CS is a promising way to improve its chelating or complexation properties. A wide variety of molecular designs to afford novel types of tailored hybrid materials are composed of natural polysaccharides and synthetic polymers. Firstly, we synthesized a comb-like pH-sensitive graft copolymer, chitosan-*graft*-poly[(2-dimethylamino)ethyl methacrylate] (CS-*g*-PDMAEMA), using atom transfer radical polymerization (ATRP) and active ester conjugation methods. The ATRP guaranteed the narrow molecular weight distribution of the side chains, which further led to a low degree of polydispersity of the copolymer aggregates. The active ester reaction took place in the mild and homogeneous solutions, and the graft ratio reached 58%. The pH-responsive

association behavior of the graft copolymer in aqueous solution was examined.

Another comb-like dual hydrophilic graft CS terpolymer: chitosan grafted with both poly[(2-dimethylamino)ethyl methacrylate] and poly(*N*-isopropylacrylamide), or simply, CS(-*g*-PDMAEMA)-*g*-PNIPAM, was synthesized by means of ATRP and click chemistry. Similarly, the ATRP ensured the narrow molecular weight distribution of the side chains. The click reaction taking place in the mild and homogeneous solution was proved to be a more efficient coupling method to obtain a high degree of grafting. The obtained graft terpolymer could aggregate into three-layer “onion-like” micelles at 25 °C when pH was above 7, whereas globular micelles with PNIPAM cores were obtained around 40 °C in an acidic solution (e.g. pH ~ 4). Moreover, switching between different types of aggregates and umimers can be reversibly achieved by properly tuning pH and temperature of the aqueous solutions.

Thereafter, serial thermo-sensitive CS(-*g*-PDMAEMA)-*g*-PNIPAM terpolymers (TCS) were prepared by ATRP and click reactions. Their lower critical solution temperature (LCST) determined by LLS was around 32.5–35.1 °C. Incorporation of CS and PNIPAM considerably decreased the cytotoxicity of PDMAEMA. Gel electrophoresis and TEM results revealed that the association and dissociation of TCS/DNA complexes could be tuned by varying temperature. The transfection level of TCS and controls were evaluated with COS7 and HeLa cells using two different reporter genes, pRL-CMV encoding luciferase and pEGFP-N1 encoding green fluorescence protein (GFP). The transfection efficiency of TCS4 incubated at 37 °C for 22 h, 20 °C for 2 h and 37 °C for 24 h increased 1–4 fold

compared to that incubated at 37 °C for 48 h. Encouragingly, at optimum N/P ratios, the transfection efficiency of TCS was comparable or superior to that of “gold-standard” PEI (25 kDa).

Finally, we report the synthesis and use of the chitosan-functionalized graphene oxide (named as CS-GO) as a nanocarrier to separately deliver a water-insoluble anticancer drug, camptothecin (CPT), and a reporter pDNA (pRL-CMV) into human cancer cell lines. Before the drug and gene delivery studies, the chemical structure, composition and morphology of the synthesized GO-CS nanocarrier were well characterized. The grafted CS branches endowed the resulting GO sheets with high solubility and stability in acidic and physiological aqueous solutions. More interestingly, GO-CS revealed a special pH-responsive association behavior as examined by DLS and zeta potential measurements. Due to the π - π stacking and hydrophobic interactions, GO-CS possesses a superior efficiency on binding CPT with a high loading content near 20 wt%. The *in vitro* drug release experiment displayed that 17.5% CPT could be released upon 72 h at 37 °C in PBS buffer. The pure nanocarrier was proved to be practically nontoxic, and the GO-CS/CPT complexes were found to afford a strong potency of killing cancer cells *in vitro*. Furthermore, as the nanocarrier is cationic, it was able to form electrostatic complexes (having particle sizes lower than 200 nm at optimal N/P ratios) with pDNA, and reasonable gene transfection efficiency was then achieved by the complexes in HeLa cells.

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2. **H. Bao**, Y. Pan, L. Li “Recent Advances in Graphene-Based Nanomaterials for Biomedical Applications”, *Nano LIFE*, *in press*.
3. **H. Bao**, Y. Pan, Y. Ping, N. G. Sahoo, T. Wu, L. Li, J. Li, L. H. Gan, “Chitosan-Functionalized Graphene Oxide for Drug and Gene Delivery”, *Small*, 7, 1569-1578 (2011).
4. **H. Bao**, L. Li, W. C. Leong, L. H. Gan, “Thermo-Responsive Association of Chitosan-graft-Poly(*N*-isopropylacrylamide) in Aqueous Solutions”, *Journal of Physical Chemistry B*, 114, 10666-10673 (2010).
5. **H. Bao**, L. Li, L. H. Gan, Y. Ping, J. Li, P. Ravi, “Thermo- and pH-Responsive Association Behavior of Dual Hydrophilic Graft Chitosan Terpolymer Synthesized via ATRP and Click Chemistry”, *Macromolecules*, 43, 5679-5687 (2010).
6. **H. Bao**, J. Hu, L. H. Gan, L. Li, “Stepped Association of Comb-like and Stimuli-Responsive Graft Chitosan Copolymer Synthesized using ATRP and Active Ester Conjugation Methods”, *Journal of Polymer Science Part A: Polymer Chemistry*, 47, 6682-6692 (2009).
7. **H. Bao**, L. Li, H. Zhang, “Influence of Cetyltrimethylammonium Bromide on Physicochemical Properties and Microstructures of Chitosan-TPP Nanoparticles in Aqueous Solutions”, *Journal of Colloid and Interface Science*, 328, 270-277 (2008).
8. **H. Bao**, L. Li, L. H. Gan, H. Zhang, “Interactions between Ionic Surfactants and Polysaccharides in Aqueous Solutions”, *Macromolecules*, 41, 9406-9412 (2008).
9. Y. Pan, **H. Bao**, N. G. Sahoo, T. Wu, L. Li, “Water-Soluble Poly(*N*-isopropylacrylamide)-Graphene Sheets Synthesized via Click Chemistry for Drug

- Delivery”, *Advanced Functional Materials*, 21, 2754–2763 (2011).
10. N. Sahoo, **H. Bao**, Y. Pan, M. Pal, M. Kakran, H. Cheng, L. Li, L. P. Tan,
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List of Symbols and Abbreviations

A_2	Second virial coefficient
ATRP	Atom transfer radical polymerization
c^*	Overlap concentration
C_2	Second critical concentration
cac	Critical aggregation concentration
C_m	Concentration of free surfactant micelles
cmc	Critical micelle concentration
CS	Chitosan
CTAB	Cetyltrimethylammonium bromide
DD	Degree of deacetylation
DLS	Dynamic light scattering
dn/dc	Specific refractive index increment
EGFP	Enhanced green fluorescent protein
GO	Graphene oxide
GPC	Gel permeation chromatography
ITC	Isothermal titration calorimetry
k	Boltzmann constant
KC	κ -Carrageenan
LCST	Lower critical solution temperature
LLS	Laser light scattering

MC	Methylcellulose
M_n	Number-average molecular mass
MTT	3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrezolium bromide
M_w	Weight-average molecular mass
n	Solvent refractive index
NMR	Nuclear magnetic resonance
PDMAEMA	Poly(2-(dimethylamino)ethyl methacrylate)
PDI	Polydispersity index
PEI	Polyethylenimine
PNIPAM	Poly(<i>N</i> -isopropylacrylamide)
q	Scattering vector
R_g	Radius of gyration
R_h	Hydrodynamic radius
ρ	Ratio of R_g and R_h
RISC	RNA-induced silencing complex
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
SLS	Static light scattering
TEM	Transmission electron microscopy
TPP	Tripolyphosphate
ΔG	Entropy change
ΔH	Enthalpy change

Chapter 1 Introduction

1.1 Background

Gene therapy has abundant potentials in both clinically applications and fundamental science research. It refers to the use of nucleic acids, such as plasmids, to increase the expression of a target protein; on the other hand, transfer RNA and oligonucleotides can be used to decrease target protein production. Studies on the delivery of therapeutic plasmid DNAs (pDNA) to defective cells have been ongoing for many years.¹⁻² In the late 1990s, RNA interference (RNAi) has emerged as a novel therapeutic pathway by which harmful genes can be “silenced” by delivering complementary short interfering RNA (siRNA) to target cell. RNAi is a process which was first found in the worm *Caenorhabditis elegans*, whereby the introduction of long double-strand RNA (dsRNA) in cells results in the degradation of homologous messenger RNA (mRNA).³⁻⁴

However, the lack of effective vectors/carriers is a major barrier for development of human gene therapy due to several dilemmas, such as vectors’ instability in body fluids, non-specificity to the target cells, degradation by enzymes and low transfection efficiency. Alexander et al.⁵ firstly proposed the concept that an infection is initiated by only using the nucleic acid of a virus, not the intact viral particle. Although these viral vectors have been applied in clinical trials due to their high transfection efficiency compared with non-viral vectors⁶, their application to the human body is often frustrated by immunogenicity, complicated production, potential infectivity and

inflammation⁷. Non-viral carriers show the advantages on (i) low immunogenicity, (ii) possibility that relatively large sequences may be condensed into small particles, (iii) good protection to gene, and (iv) easy manufacturing and possibility to be modified for target specific cells and/or diseases.

Non-viral gene delivery systems generally consist of two major categories^{2,8}: (i) lipid-based and (ii) polymer-based delivery system. The cationic liposomes such as LipofectamineTM 2000, *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethylammonium ethyl sulfate (DOTAP), cholesterol derivatives and gramicidine have been widely studied. Polymeric gene delivery systems (GDS) include: poly(L-lysine) (PLL)-based GDS; polyethylenimine (PEI)-based GDS, biodegradable polycations (poly(β -amino ester)s and poly(2-aminoethyl propylene phosphate)), polysaccharide-based GDS (cyclodextrin and chitosan), and other polycation or neutral-based GDS (e.g. poly(2-(dimethylamino)ethyl methacrylate (PDMAEMA), polyvinylpyrrolidone (PVP) etc.). Since gene/liposome complexes are relatively large in size and rapidly excreted from the body stream, cationic polymers have been proposed as an alternative approach to the development of non-viral carriers. Generally, cationic polymers may form more stable complexes with DNA than those involving lipids and they can protect DNA against nuclease degradation.

Among polymeric non-viral carriers, chitosan (CS) has been considered to be a good gene carrier candidate, because CS is known as biocompatible, biodegradable, low toxicity, and high cationic potential.⁹ CS has functional groups that allow coupling of extracellular and intracellular targeting ligands.¹⁰ However, the low

specificity and low transfection efficiency of CS must be overcome for its application in clinical trials.¹¹ Cationic CS also has the ability to complex with multivalent polyanions due to molecular ionic attraction. The CS-tripolyphosphate (TPP) nano system exhibits some attractive advantages which render them promising carriers for the delivery of peptides, proteins, oligonucleotides and plasmids.¹² Katas et al.¹³ reported that CS-TPP nanoparticles entrapped with siRNA showed a better gene silencing effect compared to the CS/siRNA complexes due to their high binding capacity and loading efficiency. Therefore, the properties of CS-TPP nanoparticles, most importantly particle size and density of surface charge, is crucial in determining gene transfection efficiency.

Although considerable improvements in gene delivery techniques using non-viral polymeric carriers (e.g. CS) have been witnessed during the last decade, the standard requirements for clinical use have not been met in terms of efficiency and specificity.¹⁻² Studies to overcome subcellular barriers, including endosomal escape and nuclear translocation, should be considered when designing polymeric carriers. In addition, gene delivery is a multi-step process, in which appropriate properties of carriers are required at each step. Therefore, rationally designed multifunctional polymeric carriers, which can overcome a series of extra- and intra-cellular barriers, are needed.

1.2 Objective and scope of the project

The main objective of this study is to explore polysaccharides based, specifically, chitosan-based novel and efficient gene delivery systems via various methods. Natural

polysaccharides are taken into account because they are biocompatible and biodegradable.

The scope of the project covers five parts of research work as described below.

(1) Study of polysaccharide-surfactant interactions

As known from the literature, ionic interactions between a negatively charged gene and a positively charged polymer are important for the gene to be condensed into a nanoparticle with the polymer. Thus, before studying the interactions between gene and polycation, we intend to examine the interactions between ionic surfactants and selected polysaccharides because (i) ionic surfactants can be used to control the fabrication of polymeric nanoparticles and the particle surface charge, and (ii) the polymer-surfactant interactions are similar to some extent to the polymer-gene interactions. The comprehensive understanding of polysaccharide-surfactant interactions could be helpful for us to select polymeric components and design workable approaches to fabrication of novel gene delivery systems. Three typical representative polysaccharides, namely methylcellulose (MC), chitosan (CS) and κ -carrageenan (KC), were selected as neutral, positively charged and negatively charged polymers. Sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) were chosen as anionic and cationic surfactants, respectively. Isothermal titration calorimetry (ITC) was used to examine the polymer-surfactant interactions.

(2) Fabrication and modulation of CS-TPP nanoparticles

It is understood from the literature that the transfection efficiency of cationic

polymers depends primarily on: (i) particle size, which determines their intracellular uptake, pathways of their uptake, intracellular trafficking and sorting into different intracellular compartments, and (ii) the intensity of particle surface charge which influences their ability to efficiently condensate gene and interact with cell membranes.¹ To control the particle size, surface charge and charge density, surfactant may play an important role. Thus, in this part, CS-TPP nanoparticles were fabricated in aqueous solutions with and without adding a cationic surfactant, CTAB. The learning from part (1) helps us to understand the effect of CTAB on the physiochemical modulation of CS-TPP nanoparticles. In this part, laser light scattering (LLS), zeta potential, and transmission electron microscopy (TEM) were used to characterize the CS-TPP particles physicochemically.

(3) Syntheses and characterizations of graft CS copolymers

Considering the CS-TPP-based gene carrier system still has a lower gene knockdown percentage than Lipofectamine¹³ and a gene carrier fabricated from original (i.e. unmodified) CS lacks desirable reactions within the CS/gene complex in an *in vivo* environment, chemical modification on CS backbones is necessary for gene delivery application. Therefore, in this part, commercial CS was chemically modified by grafting pH- or temperature-sensitive polymers prepared via atom transfer radical polymerization (ATRP) technique. The ATRP mechanism facilitates the control of growth of polymeric chains, which may result in a well-defined chain length (molecular weight) and polydispersity. We chose poly(2-dimethylamino)ethyl methacrylate (PDMAEMA) as one building block for grafting modification in order

to enhance the positive charge density along the CS backbones. PDMAEMA itself is a water-soluble cationic polymer with a much more flexible chain than CS, thus it can form more compact complexes with genes.² An improved gene transfection may result from a combination of PDMAEMA having a “proton sponge” effect and CS being biocompatible and biodegradable. The other candidate component grafted onto CS chains is poly(*N*-isopropylacrylamide) (PNIPAM), which possesses a lower critical solution temperature (LCST) in water around 32 °C.¹⁴ Subsequently, two “grafting onto” approaches were applied to conjugate PDMAEMA and PNIPAM side chains with CS backbones to achieve the objectives. These comb-like CS copolymers can “smartly” change their conformation according to the environmental changes, such as pH or temperature. Hence, the corresponding balance among electrostatic repulsion, hydrogen bonding and hydrophobic interaction was deliberately adjusted to study copolymers’ association behavior.

(4) Thermo-responsive CS-based carriers for *in vitro* gene delivery

A series of CS terpolymers (TCS), synthesized via the method demonstrated in part (3), were studied to evaluate their gene transfection efficiency. First, various concentrations of TCS were applied to complex with pDNA (i.e. pRL-CMV reporter gene) at different N/P ratios, where N/P ratio represents the molar ratio between nitrogen from TCS and phosphate from plasmid. The size, surface charge, and morphology of TCS/DNA nanoparticles were determined by DLS, zeta potential, and TEM, respectively. Nanoparticle’s stability and integrity at various ratios was investigated by agarose gel electrophoresis using ethidium bromide (EB) as the

competitive binding assay.¹¹ The cytotoxic effect of TCS was measured by determining cell viability at different polymer concentrations, and MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) is a generally used viability assay.¹³ Mammalian cell lines (e.g. HeLa) were applied to study *in vitro* transfection of TCS/DNA nanoparticles using PEI 25K and pure PDMAEMA as controls. In addition, due to the incorporation of PNIPAM in carrier materials, the level of gene transfection can be thermally controlled. By using a temperature variation protocol, i.e. incubation of the cultured cells at 20 °C for a while, the thermal influences on gene transfection efficiency was inspected.

(5) CS-graphene oxid nanocomposite for drug and gene delivery

The term multidrug resistance (MDR) is used to define a resistance phenotype where cancer cells become resistant simultaneously to multiple drugs with no obvious structural resemblance and with different molecular targets.¹⁵ Gene therapy has been reported to offer a promise to overcome MDR. Thus, combining of anti-cancer drug and gene therapies would be an effective strategy for cancer therapy.¹⁶ Under this background, we used CS-functionalized graphene oxide (named as CS-GO) as a novel nanocarrier to co-deliver a water-insoluble anti-cancer drug and a reporter pDNA into human cancer cell lines. The grafted CS branches endow the resulting GO sheets with high solubility and stability in acidic and physiological aqueous solutions. At the same time, CS is also able to form electrostatic complexes with pDNA. Due to the π - π stacking and hydrophobic interactions, GO-CS possesses a superior efficiency to bind aromatic drugs. The present successful delivery of drug and pDNA into the same cells

separately paved the way for future co-delivery studies.

1.3 Survey of the thesis

The report consists of nine chapters. The main contents of these chapters are as follows:

Chapter 1 presents a brief introduction to the whole research work. The review of interactions between polymers and surfactants, physiochemical factors affecting transfection efficiency of CS/gene complexes, and chemically modified CS as gene carriers are summarized in Chapter 2. Chapter 3 examines the interactions between ionic surfactants and polysaccharides in aqueous solutions characterized by ITC method. Afterward, Chapter 4 focuses on the influence of CTAB on the physiochemical properties and microstructures of CS-TPP nanoparticles. Chapter 5 and Chapter 6 describe the detailed synthesis processes and characterizations for CS-*g*-PDMAEMA and CS(-*g*-PDMAEMA)-*g*-PNIPAM copolymers, respectively. Their association behaviors in aqueous solutions are also investigated meanwhile. Chapter 7 reports on the thermo-responsive gene transfection properties of CS terpolymers prepared using the methods proposed in Chapter 6. In Chapter 8, we probe the possibility to use hybrid CS-GO nanocomposite as an efficient drug and gene co-delivery system. Finally, the major findings and conclusions of the present research, and recommendation for future studies are presented in Chapter 9.

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Chapter 2 Literature Review

2.1 Polymer-surfactant interactions in aqueous solutions

Interactions between water-soluble polymers and ionic surfactants are commercially important in a number of industrial applications (e.g., pharmaceutical formulations, food additives, cosmetic products, environment-friendly paints etc.). There are a considerable number of books and articles dealing with different aspects of their interactions and characteristic methods.¹⁻⁶ The interactions between polymers and surfactants can be generally described by two critical concentrations. The first concentration, critical aggregation concentration (cac), corresponds to the surfactant concentration at which surfactant molecules start to bind to polymer molecules. So, cac represents the onset for the formation of polymer-surfactant aggregation complex. The second critical concentration, C_2 , is commonly used to represent the surfactant concentration when the polymer becomes saturated with bound surfactant molecules. In addition, C_m is another critical concentration that represents the formation of free surfactant micelles in the polymer solution. For some systems free surfactant micelles start to form after the saturation concentration, and C_m is parallel to C_2 under this condition. But for some other systems C_m is less than C_2 , there is a competition between the formation of free surfactant micelles and polymer-surfactant aggregation complexes. The determinations of cac, C_m , and C_2 have been studied by Bloor and co-workers⁷⁻⁹ by using both surfactant ion selective electrodes and microcalorimetry.

The interactions between some uncharged polymers, such as poly(ethylene

oxide) (PEO), poly(propylene oxide) (PPO), poly(N-vinylpyrrolidone) (PVP), ethyl(hydroxyethyl) cellulose (EHEC) and triblock copolymers, and ionic surfactants in dilute aqueous solution have been extensively studied by isothermal titration calorimetry (ITC) in the literature.¹⁰⁻¹⁴ Wang and Olofsson¹¹ derived ΔC_p and C_2 from the calorimetric titration curves, and they found that anionic surfactant sodium dodecyl sulfate (SDS) aggregated strongly with the polymers of low hydrophobicity (PEO for instance) while some cationic surfactants (alkyltrimethylammonium bromide (RTABr)) were different. Dai et al.¹³ observed the order of binding in the triblock copolymer (PPO-PEO-PPO) and SDS system according to the hydrophobic degree of different segments.

Methylcellulose (MC) is a hydrophobically modified nonionic water-soluble polysaccharide, which has been widely used in the food and pharmaceutical industries. A MC aqueous solution is able to gel upon heating, due to the hydrophobic association of MC chains.¹⁵ Li et al.¹⁶⁻¹⁷ reported the surfactants effects on the gelation of MC using micro-DSC. Above the critical micellization concentration (cmc), both CTAB and SDS show a salt-in effect on MC, which shifts the sol-gel transition of MC to higher temperature. However, the effects of SDS and CTAB on MC are different. SDS has a weaker salt-in effect on MC than CTAB because the cmc of SDS is about 9 times that of CTAB at room temperature. Application of ITC to the study of interactions between MC and surfactants has not been found in the literature.

In the last two decades, interactions between polyelectrolytes (especially biomacromolecules such as polysaccharides) and oppositely charged surfactants have

attracted a great deal of interest due to their applications in the biological industry.¹⁸⁻²⁰ As a cationic polysaccharide, chitosan (CS) has many applications dealing with the interactions of CS with anionic molecules such as anionic phosphates, lipids, or proteins.²¹⁻²³ The influences of pH, salt, and temperature on interactions between CS and SDS have been studied by Thongngam and McClements²⁴⁻²⁵ using ITC. The strong electrostatic binding between CS and SDS was observed at pH = 3.0 at which CS was fully protonated. The amount of SDS bound to CS at saturation was weakly affected by the presence of NaCl and temperature variation.²⁵ Carrageenans (CARs) are a family of water-soluble and linear sulfated polysaccharides extracted from red seaweeds. In the family of carrageenan, there are three main members: κ , ι , and λ . As the first member, κ -carrageenan (KC) has one sulfate group within every disaccharide repeating unit ideally (see chemical structure in Section 3.1.3). Filipović-Vinceković and coworkers^{20,26} have investigated the interactions between dodecylammonium chloride (DDAC) and CARs by surface tension, conductometry, light microscopy, light scattering etc. They found that the effect of CAR concentration on the cac and cmc of surfactant was very weak. The increase in the charge density of CAR (from κ to ι and λ CARs which have more sulfate groups per disaccharide unit) increased cac and the cac/cmc ratio and shifted the precipitation toward higher concentration.²⁶

2.2 Physicochemical factors affecting transfection efficiency of CS/DNA complexes

The numerous studies of CS for gene delivery, its efficiency and safety have been found from the literature.²⁷⁻²⁹ The optimum conditions for transfection efficiency

of CS/DNA are still in debate, and shall be elucidated for their practical application as gene carriers. Herein, the effect of molecular weight (MW) and degree of the deacetylation (DD) of CS, pH and N/P ratio on the transfection efficiency *in vitro* will be discussed. In the last section, factors affecting gene-silencing activity of CS/siRNA complexes will be reviewed to highlight the similarities and differences with DNA therapies.

2.2.1 Molecular weight (MW) of CS

The effect of MW of CS on the complex formulation with DNA has been attributed to a chain entanglement effect. CS with high MW can more easily entangle free DNA once the initial electrostatic interaction has occurred. As a matter of fact, Huang et al.³⁰ reported that low MW CS was less efficient at retaining the DNA upon dilution, and consequentially, less capable of protecting the condensed DNA from degradation by DNase and serum components, and resulted in low transfection efficiencies. On the other hand, in a recent study by Köping-Höggard et al.³¹, CS oligomer (18 monomer units)/DNA complexes formed at a N/P ratio of 60 were shown to have higher transfection efficiency than high MW (162 kDa) CS/DNA complexes prepared at N/P ratio of 2.4, due to the better capability to release DNA from the complexes; while the heptamer of CS (MW = 1.3 kDa) did not show any gene expression³². Therefore, a fine balance must be achieved between extracellular DNA protection (better with high MW) and efficient intracellular unpackaging (better with low MW) to obtain high transfection efficiency of CS.³¹

2.2.2 Degree of deacetylation (DD) of CS

DD influences the characteristics of CS, such as solubility, charge density, crystallinity, and degradation etc. Most CS/DNA complexes studied have used highly deacetylated CS (DD above 80%).³³⁻³⁴ Huang et al.³⁰ once found that A549 cellular uptake of the CS/DNA nanoparticles was significantly reduced by decreasing the DD of the CS, which resulted in low transfection efficiency, indicating that cellular uptake of nanoparticles is mediated by electrostatic interactions with the cell membrane. Kiang et al.³⁵ investigated the effect of DD of CS acetylated with acetic anhydride on the efficiency of gene transfection, and their results indicated that DNA binding efficacy decreased with decreasing DD. Low DD resulted in decreased overall luciferase expression levels in cells owing to destabilization of the nanoparticles caused by the bulky acetyl groups in the polymer chains, although the luciferase expression levels for CS with DD of 70% and 62% are up to two orders of magnitude higher than that of CS with DD of 90% *in vivo*, reflecting the differences in behavior between *in vivo* and *in vitro*.³⁵ On the other hand, Lavertu et al.³⁶ reported that the maximum expression levels could be obtained by simultaneously lowering MW and increasing DD, or lowering DD and increasing MW, suggesting a predominant role of particle stability in determining efficiency through co-operative electrostatic binding.

2.2.3 pH environment

The transfection efficiency of CS/DNA complexes was largely dependent on the pH value of the cell culture medium because protonated amino groups in CS facilitate its binding to negatively charged DNA, and condense into nanoparticles

through phase separation. Considering that the pK_a value of CS is around 6, the primary amines in CS become positively charged at acidic pH (below 5.5). Based on the literature, a pH slightly lower than 7 is optimal to achieve a good balance between DNA association and dissociation.

Initially, Sato et al.³² compared CS's transfection efficiency on A549 cells at pH 6.9 and 7.6. They found that the transfection efficiency at pH 6.9 was higher than that at pH 7.6, because the CS/DNA complexes at pH 6.9 are positively charged and can bind to the negatively charged cell membranes through electrostatic interaction. After that, Lavertu and coworkers³⁶ reported that the transfection efficiency at pH 6.5 with CS (MW = 10 kDa, DD = 92% and N/P ratio = 5) was higher than that at pH 7.1 and equivalent to FuGENE6, a highly efficient commercial carrier. Systematically, Zhao et al.³⁷ investigated the pH effect of transfection medium on the transfection efficiency of chondrocytes. It was shown that the maximum expression efficiency was achieved at pH 6.8–7.0, and the transfection efficiency decreased dramatically when the pH of the medium increased to 7.4. This finding may be attributed to the dissociation of free plasmid from the complexes at the higher pH.³⁸ By contrast, at a more acidic pH, the transfection efficiency decreases due to very strong electrostatic interactions that ultimately slow down DNA release, as reported by Liu's group.³⁹

2.2.4 Charge ratio (N/P ratio)

The charge ratio (N/P) is given by the stoichiometry between CS's nitrogen and DNA's phosphate. Ishii et al.³⁸ firstly concluded that the transfection efficiency of the CS/plasmid complexes increased at charge ratios of 3 and 5, and decreased at

higher ratios of complexes owing to relationship between cell uptake and transfection efficiency. Lavertu et al.³⁶ also discussed that for the same DD, a lower MW CS requires a higher N/P ratio to completely condense DNA, whereas a lower DD requires a higher N/P ratio to completely condense DNA at equal MW. If the N/P ratio is too low, physically unstable nanoparticles will be obtained thus leading to very poor transfection. However, if complexes are prepared at a too high N/P ratio, the transfection expression may also be suppressed, suggesting that there is an optimal range for the N/P ratio specific to the CS used.

2.2.5 Factors affecting gene silencing efficiency of CS/siRNA complexes

Liu et al.⁴⁰ studied the influences of CS's MW, DD and N/P ratio on particle size of CS/siRNA complex. They summarized that higher MW and DD are favorable for the formation of nanoparticles, since higher MW CS molecules are longer and more flexible while a higher DD increases the positive charge density along the CS polymer chain. They also found that CS with lower DD but higher MW formed nanoparticles by compensation of low charge density on the long chain. Longer chain CS molecules may also favor the hydrophobic interactions or hydrogen bonds between the sugar residues of CS and the organic bases of the nucleotide to form nanoparticles. In contrast with low MW CS/DNA nanoparticles, which are stable due to compensation of longer DNA chain lengths with shorter CS chains, CS with lower MW (< 10 kDa) cannot complex siRNA into stable and compact particles. At the same time, CS with low DD has less electrostatic interaction with siRNA, and particles formed are unstable. Thus, the authors concluded that higher MW, higher

DD and high N/P ratio are crucial requirements for making stable and compact CS/siRNA nanoparticles.

Based on a recent study by atomic force microscopy (AFM), the interaction strength between siRNA and CS was pH dependent and the adhesive interactions decreased as the pH was increased from 4.1 to 6.1, 7.4, and 9.5, exhibiting distinct multimodal distributions of the interaction forces between them at acidic pH and only negligible adhesive forces were observed at neutral or high pH. The strong pH dependence of siRNA–CS interactions can provide a convincing rationale for complex formation and nanoparticle stability under low acidic conditions.⁴¹

Furthermore, Katas and Alpar⁴² used four types of medical grade CS, namely, CS hydrochloride and CS glutamate which each has two different molecular weights (MWs) for the delivery of siRNA. Three methods of siRNA association were studied: simple complexation, ionic gelation and adsorption of siRNA onto the surface of CS nanoparticles. This study showed that lower CS MW and concentration are favorable for the formation of smaller particle sizes. In addition, CS glutamate leads to smaller particles than CS hydrochloride irrespective of MW.⁴² Moreover, Howard et al.⁴³ examined the efficiency of CS nanoparticles in delivering siRNA *in vivo* and *in vitro*, concluding that particle sizes of CS/siRNA complexes depended on the N/P ratio. An optimum concentration of CS must use higher N/P ratio. Throughout the CS concentrations, a decrease in N/P ratio increased the particle size due to siRNA bridging between CS chains at increased siRNA levels, leading to greater CS incorporation and possible inter-particle aggregation.⁴³

The shorter length and linearity of siRNA is the prime feature motivating researchers to modify conventional CS based DNA delivery system. The interaction between CS and siRNA by simple mixing is apparently weak, and CS and siRNA are easily dissociated from each other. To solve this issue, Katas and Alpar⁴² have added tripolyphosphate (TPP) as a gelling agent to a CS solution to obtain smaller particle sizes, by decreasing the pH value to 4.5. Due to the increased number of protonated amino groups at low pH, CS could efficiently complex with siRNA and TPP to produce smaller and compact nanoparticles. The in situ entrapping siRNA by the CS-TPP nanoparticles formed in the ionic gelation process gives better gene silencing compared with CS particles prepared by simple complexation or adsorption of siRNA on preformed CS-TPP nanoparticles, due to poor protection of siRNA from nuclease enzyme.

2.3 Chemical modification of CS as a gene carrier

2.3.1 Hydrophilic modification

The bulk of the plasma clearance after administration of DNA *in vivo* is due to uptake by lung endothelia, fixed macrophages in the liver sinusoids and in the spleen. The hepatic clearance of naked DNA is very close to the plasma flow rate through this organ, indicating that DNA is cleared substantially on first-pass through non-parenchymal cells, following interaction of DNA with scavenger receptors for polyanions.⁴⁴ Thus, it is necessary to neutralize the polyanionic charge of DNA to avoid uptake by non-parenchymal cells.⁴⁵ When the DNA forms complexes with cationic polymers, the complexes show short plasma circulation times with rapid

hepatic uptake and accumulation or deposit in organs. Aggregation of cationic particles from cationic polymer/DNA complexes can occur following interaction with blood components, albumin, for example. The binding of albumin causes aggregation of particles in the blood stream by reducing their zeta potential, hence reducing the charge repulsion between particles. Therefore, it is necessary to prevent unwanted interactions between particles and the dynamic environment of the blood circulation by introducing hydrophilic segments on the cationic particles.

Allen⁴⁶ reported that the incorporation of flexible hydrophilic polymer into gene delivery systems produces a steric barrier to interaction with proteins and phagocytes and prolongs plasma circulation time. Park et al.⁴⁷ incorporated dextran as a hydrophilic group into galactosylated CS (GC) to increase stability of complexes. The turbidity experiments shows GC/DNA complexes were constant or slightly decreased with time whereas the turbidity of the CS/DNA complexes gradually increased or the complex drastically precipitated at the early stage of complexation, indicating that dextran enhanced stability. The coupling of dextran into CS seems to be responsible for protecting the complexes from self-aggregation and precipitation.

As we know, extravasation of particles is a function of their size and the permeabilities of the different vascular layers that they encounter. For example, access to the liver probably requires particles' diameter smaller than 100 nm. The particles size and surface charge depend subtly on the hydrophilic groups of the complexes. Park et al.⁴⁸ introduced poly(ethylene glycol) (PEG) as a hydrophilic group into the GC to render better solubility and to reduce the particle sizes of the

complexes. The particle sizes of GCP/DNA complexes decreased with increasing charge ratio of GCP to DNA and had a minimum value at around 27 nm at the charge ratio of 5, whereas GC/DNA complexes without PEG showed a tendency to aggregate to 1 μ m. It was proposed that hydrophilic PEG chains at the surface of complex as neutral charge induce the maximal compaction of DNA and prevention of aggregation of neutralized complexes. A lot of research has focused on the “PEGylation” of PEI to increase stability and to improve the safety of PEI/DNA complexes⁴⁹⁻⁵⁰, reflecting much higher transfection efficiency of the PEI/DNA complexes compared with those of CS/DNA *in vitro* and *in vivo*.⁵¹ Nevertheless, high MW PEIs show low colloidal stability and high *in vivo* toxicity.⁵²

Park et al.⁵³ have incorporated another hydrophilic polymer, poly(vinyl pyrrolidone)(PVP), into GC to prevent albumin from interacting with the complexes. The effect of albumin on the surface charge of GC-graft-PVP (GCPVP)/DNA and PEI/DNA complexes is contrasted: GCPVP/DNA complexes had a constant surface charge regardless of the amount of albumin added whereas the zeta potential of PEI/DNA complexes at charge ratios of 1 and 3 drastically decreased to negative, indicating that the PVP groups prevented albumin from interacting with the GCPVP/DNA complexes.

The preparation of trimethylated CS (TMC) by introducing quaternization of the CS backbone to make the CS soluble over a wide range of pH was reported by Kean et al.⁵⁴, and they found that the TMC was significantly less toxic than PEI 25K. TMC/pGL3 luciferase plasmid DNA complexes were able to transfect MCF-7 cells

with higher efficiency than PEI 25K (by 16-50 fold according to the degree of trimethylation owing to the increased positive charges). Satoh et al.⁵⁵ prepared and evaluated 6-amino-6-deoxy-CS as a gene carrier, which was soluble in water at neutral pH. They found that the C-6 positions of CS were successfully modified in a highly regioselective manner and the transfection efficiency of the modified CS/DNA complexes to COS-1 cells was superior to CS, with good cell viability as well.

2.3.2 Hydrophobic modification

Hydrophobic groups in the non-viral carriers are expected to increase transfection efficiency by changing complex interactions with cells, such as adsorption on cell surfaces and cell uptake.⁵⁶ Furthermore, these hydrophobic sections in the carriers also can help dissociating polymer/DNA complexes, thus facilitating release of DNA that is strongly bound through ionic interactions between cationic parts of the polymer and the anionic phosphates of DNA. These favorable characteristics of the hydrophobic units may bring a higher transfection efficiency of CS than those polymeric carriers only with ionic interactions.

The complex formation of deoxycholic acid-modified CS (DAC) with DNA had a strong dependence on the size and structure of CS self-aggregates and significantly influenced the transfection efficiency of COS-1 cells (up to a factor of 10), although the transfection efficiency of the complexes remarkably decreased in the presence of serum.⁵⁷ On the other hand, deoxycholic acid modified CS oligomer (DACO) nanoparticles showed superior gene condensation compared to unmodified CS oligomer and great potential for gene carrier with the high level of gene

transfection efficiencies due to the amphiphilic nature of the DACO, even in the presence of serum.⁵⁸ The transfection efficiency of the complexes was highly dependent on the degree of substitution and medium pH.

Liu et al.⁵⁹ prepared *N*-dodecylated CS (NDC) and complexed it with DNA. The incorporation of hydrophobic dodecyl group on CS enhanced the thermal stability of DNA. They also prepared alkylated CS (ACS) by modifying CS with alkyl bromide, and used it to transfer plasmid-encoding CAT into C₂C₁₂ cell lines. When extending the alkyl side chain in the ACSs, the transfection efficiency was increased and leveled off after the number of carbons in the side chain exceeded eight. They proposed that the higher transfection efficiency of ACS is attributed to the increasing entry into cells, which is facilitated by hydrophobic interactions and easier unloading of DNA from ACS carriers owing to the weakening of electrostatic attractions between DNA and ACS.⁶⁰ As for the alkyl side chains, Thanou et al.⁶¹ prepared trimethylated CS oligomers and investigated the transfection efficiency of the modified CS/DNA complexes. The results indicated that TMC/DNA complexes seemed to be superior to oligomeric CS transfection in COS-1 cells.

Yoo and co-workers⁶² prepared 5 β -cholanic acid modified glycol CS (CGC) for gene delivery. The CGC/DNA nanoparticles were prepared by a hydrophobic interaction between CGC and CTAB hydrophobized DNA. Increasing with the CGC content, the encapsulation efficiencies of DNA increased while the size of the nanoparticles decreased. The increased GCG contents also led to increased *in vitro* transfection efficiencies in the presence of serum. Similarly, Hu et al.⁶³ coupled

stearic acid (SA) as a hydrophobic group to CS oligomer (CSO) and studied the transfection efficiency in A549 cells. The results indicated that the transfection of SA-CSO/DNA complexes increased with the post-transfection time over 76 h to approach that of LipofectamineTM 2000, whereas that of Lipofectamine/DNA rapidly decreased after 24 h due to the cytotoxicity of Lipofectamine. On the other hand, hydrophobic PEIs reduced transfection efficiency due to the reduced basicity although dendrimers having hydrophobic amino acid residues increased transfection activity owing to the synergy of the “proton sponge” effect induced by the dendrimer tertiary amines, and hydrophobic interaction by the amino acid residues.⁶⁴⁻⁶⁵

2.3.3 pH-sensitive modification

Generally, several steps are required for efficient gene transfection, including gene complexation, cellular uptake of the complexes, release of gene or complexes from endosomes, release of gene from the carriers, and permeation into the nucleus.⁶⁶ Inefficient release of the polymer/gene complex from endocytic vesicles into the cytoplasm is one of the primary reasons for poor gene delivery.

Imidazole-containing polymers using histidine has been reported to act as a “proton sponge” and enhance release of the complex into the cytoplasm following endocytosis.⁶⁷ Recently, Kim et al.⁶⁸ modified CS with urocanic acid (UA) bearing an imidazole ring, which can play the crucial role in endosomal rupture according to a proton sponge mechanism. Figure 2.1 shows the synthetic method for the urocanic acid modified CS (UAC). The transfection of 293T cells by UAC/DNA complexes *in vitro* confirmed that the transfection efficiency increased with increasing the N/P ratio

and substitution degree of UA groups, reflecting the higher buffering capacity in the endosomal compartment, which leads to positively charged ions being trapped by the imidazole rings, and enhances osmolarity, subsequent endosomal rupture and escape into the cytoplasm. This mechanism is similar to that of PEI, which is the most used non-viral carrier as a control, although the transfection efficiency increment against 293T cells by the UAC/DNA complexes is still lower than that of PEI.

Figure 2.1 Synthetic scheme of UAC. (Source: Ref.⁶⁸)

Kim et al.⁶⁹ reported that addition of PEI to water-soluble CS (WSC)/DNA and GC/DNA complexes dramatically increased transfection efficiency (10 to 1000 fold) in different cell lines, most probably owing to induction of proton sponge effect by PEI. In addition, they also found that the GC/DNA complex still had the galactose-mediated gene transfer property even after combination with PEI. Wong and co-

workers⁷⁰ prepared PEI-*graft*-CS using cationic polymerization of aziridine with water-soluble oligo-CS as shown in Figure 2.2. The result relied on a combination of an oligo-PEI having a proton sponge effect, and CS being biocompatible and biodegradable. It was concluded that PEI-*g*-CS had a lower cytotoxicity and higher transfection efficiency than that of PEI 25K both *in vitro* and *in vivo*. Jiang et al.⁷¹ also synthesized CS-*graft*-PEI by an imine reaction proceeded between periodate-oxidized CS and amine groups of low MW PEI. They reported that the CS-*g*-PEI/DNA complexes showed higher transfection efficiency and lower cytotoxicity than PEI 25K in HeLa, 293T and HepG2 cell lines at the high N/P ratio, attributing to the buffering effect of PEI. The transfection efficiency of the complexes was comparable to that of LipofectamineTM 2000. By far, among the studied CS derivatives, the incorporation of low MW PEI into CS showed the highest transfection efficiency with low cytotoxicity *in vitro*.

Kiang et al.⁷² added poly(propyl acrylic acid) (PPAA) to the CS/DNA complexes in order to enhance the transfection efficiency of the complexes. PPAA, as a highly pH-sensitive polymer, exhibits the maximum membrane disruption ability at $\text{pH} < 6.0$ and results in the disruption of the endosomal membrane to release the vesicle contents into the cytosol.⁷³ They reported that the introduction of PPAA to CS/DNA complexes enhanced gene expression in both HEK293 and HeLa cells compared to CS nanoparticles alone.⁷²

Figure 2.2 Synthetic scheme of PEI-g-CS. (Source: Ref.⁷⁰)

2.3.4 Thermo-sensitive modification

Stimuli-responsive polymers exhibit fast reversible physical changes in response to external stimuli such as pH, temperature, ionic strength, light, solvent, electrical or magnetic field. Among stimuli-responsive polymers, poly(*N*-isopropylacrylamide) (PNIPAM) is a well-known thermo-sensitive polymer and has been extensively used as a drug carrier, because of its lower critical solution temperature (LCST) around 32 °C.⁷⁴⁻⁷⁵ Sun et al.⁷⁶ coupled a carboxyl-terminated *N*-isopropylacrylamide/vinyl laurate (NIPAM/VL) copolymer with CS (PNVLCS) and investigated the gene expression of PNVLCS/DNA complexes in C₂C₁₂ cells according to temperature change. They found that the transfection efficiency of

PNVLCS/DNA complexes was improved by dissociation of the gene from the carrier when temporarily reducing the culture temperature to 20 °C. By contrast, naked DNA and Lipofectamine could not demonstrate such thermo-responsive gene transfection. Also, thermo-responsive CS-*graft*-PNIPAM⁷⁷ and hydroxybutyl CS⁷⁸ had also been applied for the culture of mesenchymal stem cell.

2.3.5 Specific ligand modification

CS can be immobilized on specific surfaces when it carries some ligands. The specific ligands in the CS enable specific binding to the receptors of the cells and enhance the transfection efficiency. The mechanism of transfection efficiency by the several specific ligands in CS is elaborated in the following.

2.3.5.1 Galactose ligand

Parenchymal liver cells, hepatocytes, are useful target cells for gene delivery because they can act as a host of post-translational modification that may be required for the activity of certain gene products. The liver is still an attractive target tissue for gene therapy due to its large size, metabolic capacity and rich blood supply that can be useful for the delivery of genes to the liver as well as for the distribution of gene products from the liver to the systemic circulation. Furthermore, mammalian hepatocyte is the unique cell that possesses large number of high-affinity cell-surface receptors that can bind asialoglycoproteins (ASGP).⁷⁹ The asialoglycoprotein receptors (ASGP-R) are known to be present only on hepatocytes at a high density of 500,000 receptors per cell, and retained on several human hepatoma cell lines. These receptors cannot only bind to galactose-bearing ligands, but can internalize them

within membrane-bound vesicles or endosomes.

TMC bearing antennary galactose residues as the CS derivatives was examined for specific targeting to HepG2 cells after complexing with DNA.⁸⁰ The conjugates had a tendency to increase the cellular recognition ability with increasing galactose residue, indicating that the conjugates were specifically internalized via the galactose receptor on the HepG2 cells. Gao et al.⁸¹ reported that the galactosylated low MW CS carrier transfected gene into HepG2 selectively via the receptor-mediated endocytosis pathway, and the transfection efficiency was influenced by the galactosylation degree and N/P ratio although the modified CS had relatively low cytotoxicity. Kim et al.⁸² incorporated galactose bearing lactobionic acid to water-soluble CS for hepatocytes specificity because WSC dissolves in water while decreases the cytotoxicity induced by acetic acid. Recently, Hashimoto et al.⁸³ synthesized lactosylated CS by reductive alkylation in the presence of sodium cyanoborohydride for hepatocytes DNA delivery. They found that the lactosylation of CS increased the physicochemical stability of the DNA complex, and the lac-CS/DNA complexes exhibited high transfection efficiency for hepatocytes having ASGP-R and the accumulation of DNA complexes in the late endosome/lysosome.

2.3.5.2 Transferrin ligand

Transferrin receptor, found on many mammalian cells, is responsible for iron import to the cells. It is known that the transferrin, as a ligand, could efficiently transfer small MW drugs, bioactive macromolecules, and liposomes through a receptor-mediated endocytosis mechanism. Mao et al.³³ have conjugated transferrin to

CS nanoparticles surface through a disulfide bond because reversible disulfide linkage would allow the cleavage of the transferrin moiety after cellular uptake, and in turn facilitate the nanoparticles disaggregation and the DNA release. The transferrin conjugation showed 4-fold increment in their transfection efficiency in HEK293 and HeLa cells compared with those unmodified nanoparticles. *N*-(2-hydroxy-3-trimethylammonium)propyl CS chloride was ionically cross-linked by TPP to obtain sub-200 nm nanoparticles and apo-transferrin known to enter cells via receptor-mediated endocytosis was conjugated to the nanogels for targeted pH-mediated intracellular release of cancer drug.⁸⁴ The results indicated that bioconjugated nanogels were efficiently internalized into the HeLa cells through receptor-mediated endocytosis.

2.3.5.3 Folate ligand

Folic acid (FA) is one kind of ligand for targeting cell membrane and allows nanoparticles endocytosis via the folate receptor (FR) for higher transfection yields. Folate has a high affinity to bind to its receptor ($K_D < 1$ nM), and its small size facilitates specific cell targeting. Mansouri et al.⁸⁵ incorporated FA to CS through amide linkage between amino groups of CS and carboxylic groups of FA for folate-mediated endocytosis. Lee and coworkers⁸⁶ conjugated FA as a targeting ligand to CS to specifically deliver DNA to FR-over expressing cancer cells. They found that FA-conjugated CS exhibited a significantly increased gene transfer potential in FR-over expressing cancer cells as compared to unmodified CS.

2.3.5.4 Mannose ligand

The T-cells and the B-cells are essential for efficient gene delivery systems used in antigen presenting cells (APCs) that can activate the effector cells of the immune defense. For effective manipulation of a wide range of immune functions, the regulation of immunity is an important goal in prevention and treatment of viral infections, cancer and autoimmune diseases. The APCs such as macrophages and immature dendritic cells (DCs) can express high levels of mannose receptor that are used for endocytosis and phagocytosis of a variety of antigens that expose mannose.⁸⁷

Kim et al.⁸⁸ have fabricated mannosylated CS (MC) to induce the receptor mediated endocytosis for targeting into APCs. MC/DNA complex indicated higher transfection efficiency compared with WSC/DNA complex on Raw264.7 macrophage cell line and the transfection efficiency of the MC/DNA was decreased in the presence of mannose, being attributed to the receptor-mediated endocytosis mechanism. Hashimoto et al.⁸⁹ coupled formylmethyl mannoside prepared by reductive ozonolysis of allyl mannoside with CS by reductive alkylation in the presence of sodium cyanoborohydride. The results showed that mannose-modified CS/DNA complexes had much higher transfection efficiency than CS/DNA, and the recognition of the man-CS/DNA complexes depended on the substitution degree of the mannose residues in CS.

2.4 Summary for problem statement

Although gene therapy holds tremendous promise in many genetic and acquired diseases treatments, its future is contingent upon the discovery of safe and

effective carriers of genetic material. Crucial to successful delivery of the gene to the cell is the carriers' ability to protect the genes against enzymatic degradation. A well-designed carrier will also promote cellular uptake and intracellular release of the nucleotides.

Recent studies have indicated that even for the same CS-based gene carrier, different results can be obtained for siRNA delivery compared to that of DNA delivery. Contrast with DNA's several kilo base pairs, 21-23 bp siRNA segments behave as rigid rods which are not likely to be condensed into small, nanometric particles when complexed with cationic carriers. As a promising candidate of non-viral siRNA carriers, CS shows outstanding biocompatibility, but the electrostatic repulsion between charges along a CS chain greatly decreases its local flexibility. Therefore, their disorderly interactions may result in incomplete encapsulation and/or undesirably large complexes. It is generally accepted that the size limit for non-specific cellular uptake is 150 nm.⁹⁰ In addition, other factors such as zeta potential and ionic stability are also directly correlated with transfection efficiency. For example, nanoparticles bearing a positive surface charge are believed to facilitate uptake by negatively charged cell membranes. In summary, to fabricate smaller, more compact CS nanoparticles with a high surface charge density is the first and unescapable challenge.

By applying the knowledge learnt from the literature on the studies of DNA delivery problems, we wish to take some challenges in CS using carriers for gene delivery. In this work, we will employ both physical and chemical methods to

modulate CS-based delivery systems in order to improve their *in vitro* gene transfection efficiency. Of course, successful *in vivo* gene delivery requires a carrier that is capable of performing multiple functions, each at an appropriate timing during the delivery processes including extracellular stage and endosomal release. Full understanding of these barriers will help design and fabricate more efficient gene carrier systems.

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Chapter 3

Interactions between Ionic Surfactants and Polysaccharides

3.1 Introduction

In this chapter, methylcellulose (MC), chitosan (CS) and κ -carrageenan (KC) are chosen as the representatives of nonionic, cationic and anionic polysaccharides respectively. Their interactions each with sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) (serving as anionic and cationic surfactants respectively) are systematically studied using isothermal titration calorimetry (ITC).

3.1.1 Polysaccharides

3.1.1.1 Chitosan

Chitosan (CS), α (1 $\rightarrow$ 4) 2-amino-2-deoxy- β -D-glucan, is a naturally occurring polysaccharide and its commercial forms are essentially produced from N-deacetylation of chitin. As the second most abundant polysaccharide in nature, chitin is found in the exoskeleton of crustacea, insects, and some fungi. The chemical structure of chitosan is shown in Figure 3.1.

Figure 3.1 Chemical structure of chitosan.

Two kinds of chitosan are employed in this thesis: medium molecular weight chitosan ($M_n \sim 120$ kDa) with a nominal degree of deacetylation, 75–85 %, which was

purchased from Sigma-Aldrich; and chitosan oligomers ($M_n \sim 3, 5, 10$ kDa respectively) with a nominal ~85% degree of deacetylation, which were obtained from Haidebei Marline Bioengineering Co., Ltd., China. Since the commercial chitosan materials in the form of fibril flakes contained some insoluble impurities, a modified purification method according to Gan et al.¹ was utilized. The raw chitosan material was dissolved in an acetic acid solution under vigorous stirring. A primrose yellow colored solution was obtained and then filtered several times using a Hartley funnel to remove the insoluble residues. 1.0 M NaOH solution was added dropwise into the filtrate to adjust its pH value to 7.5–8.0, letting chitosan precipitate. The precipitated chitosan was centrifuged and washed repeatedly using deionized water to remove the excess salt (NaAc). Finally, the wet product was freeze-dried using the Alpha 1-2 LD freeze drier (Martin Christ, Germany) at -57 °C for 24 h to obtain the purified chitosan flakes.

3.1.1.2 Methylcellulose

Figure 3.2 illustrates the chemical structure of methylcellulose (MC). Any of the three hydroxyl groups in one cyclo-structured glucose unit could be substituted by a $\text{CH}_3\text{O}-$ group via methylation reaction. An average degree of substitution (DS) is defined and the maximum value of DS is 3, meaning that all the hydroxyl groups in each glucose unit have been substituted.

Figure 3.2 Chemical structure of methylcellulose.

In this study, methylcellulose (trade name of SM4000) was provided by Shinetsu Chemical Co., Ltd., Japan. According to the product specification: SM4000 has the weigh-average molecular weight around 310 KDa determined by light scattering; and its DS is 1.8. The material was directly used as received, since Li et al.²⁻³ has verified its purity by micro-DSC tests. Methylcellulose was firstly added into hot water to achieve a good dispersion when preparing the solutions, and the partially dissolved samples were put into a refrigerator (4 °C) to get a clear phase.

3.1.1.3 Kappa-Carrageenan

The carrageenans (CAR) are a family of linear sulfated galactans extracted from various species of marine red algae. κ -Carrageenan is produced by alkaline elimination from μ -carrageenan isolated mostly from the tropical seaweed *Kappaphycus alvarezii* (also known as *Eucheuma cottonii*). Figure 3.3 shows the chemical structure of κ -carrageenan:

Figure 3.3 Chemical structure of κ -carrageenan.

This material was obtained from Sigma-Aldrich and used as received, whose

viscosity ranges 5–25 mPa.s (0.3% in H₂O, 25 °C). To prepare the aqueous solutions, κ -carrageenan was dissolved in hot water (maximum 5 mg/mL) and cooled down to room temperature. Solutions were kept at 4 °C to avoid the degradation of κ -carrageenan, and they should be disposed of when precipitate occurs.

3.1.2 Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) measures the differential enthalpy changes isothermally while one component is titrated into another. From the analysis of the ITC thermogram, various thermodynamic parameters, such as enthalpy and heat capacity could be obtained. The isothermal microcalorimetric experiments were carried out using an isothermal titration calorimeter (ITC) (Microcal Inc., MA). The details of ITC were described elsewhere.⁴ The microcalorimeter consists of a reference cell and a sample cell of 1.35 ml, with both cells insulated by an adiabatic shield (see Figure 3.4).

Isothermal titration calorimetry (ITC) is a powerful tool for studying thermodynamic interactions between a polymer and a surfactant in an aqueous solution. Except for the ITC studies on interactions between CS and SDS which can be found in the literature,⁵ this is the first report in which the polymer-surfactant pairs of MC-SDS, MC-CTAB, CS-CTAB, KC-SDS, and KC-CTAB are studied by means of ITC. A comprehensive understanding of the characteristic molecular interactions within these six polymer-surfactant systems will be given.

Figure 3.4 Schematic diagram of isothermal titration calorimetry.

3.2 Experimental

The titration was carried out by stepwise injection of surfactant solutions from a 250 μl injection syringe into the sample cell filled with either water (or buffer) or dilute polymer solutions. The syringe is tailored-made so that the tip acts as a blade-type stirrer to ensure an optimum mixing efficiency at 400 rpm. Using the interactive software, an automatic injection schedule was made by setting the number of injections, volume of each injection, and time between injections. The time interval between coterminous injections was set to 240 sec. The heat evolved or absorbed by each injection in the course of titration was directly measured by the microcalorimeter.

The concentrations of all the polymer solutions as titrate were fixed at 0.1 wt% for comparative results. In addition, the effect of polymer concentration was studied for some selected polymer-surfactant pairs (MC-CTAB, KC-SDS, and KC-CTAB) where polymer concentrations of 0.05 and 0.25 wt% were used. All titrations were performed at a constant temperature of 25.0 ± 0.1 °C. The low concentrations (≤ 0.25 wt%) of the polymer solutions and the moderate temperatures applied resulted in a relatively low viscosity at any point of titration, which did not affect the mechanical stirring of the microcalorimeter.

3.3 Results and discussion

3.3.1 Interaction between MC and SDS

The calorimetric titration curve (filled squares) for the addition of 200 mM SDS solution to a 0.1 wt% MC solution at 25 °C is shown together with the thermogram of cell feedback (CFB) in Figure 3.5. The corresponding curve from the dilution of SDS in water (open squares) is included too. The SDS concentration 200 mM is about 24 times the cmc (8.3 mM^{6-7}) of SDS at 25 °C so that the 200 mM SDS solution is a micellar solution. When the micellar solution of SDS is titrated into the sample cell filled with deionized water, the micelles of titrated SDS start dissociating to produce monomers until the concentration of SDS in the sample cell reaches its cmc. The cmc (about 8.3 mM) of SDS is shown as the surfactant concentration for the maximum of the dilution curve or the slope of the differential curve becomes zero in the inset of Figure 3.5b. When the SDS concentration exceeds the cmc, the micelles will only be diluted. Thus, the thermodynamic properties of the demicellization of SDS can be obtained from the ITC curve.

(a)

(b)

Figure 3.5 Calorimetric titration of 200 mM SDS into 0.1 wt% MC at 25 °C. (a) Thermogram showing cell feedback (CFB) versus time. (b) Differential enthalpy curves versus concentration of SDS (■), (inset) difference plots of the calorimetric curves. Dilution of SDS (200 mM) in water at the corresponding temperature represents as (□).

ΔG_{mic} and ΔS_{mic} can be calculated from⁸

$$\Delta G_{\text{mic}} = (1 + K)RT \ln(\text{cmc}) \quad (3-1)$$

$$\Delta G_{\text{mic}} = \Delta H_{\text{mic}} - T\Delta S_{\text{mic}} \quad (3-2)$$

where K is the micellar change fraction and equals 0.85 for SDS.⁹⁻¹⁰ At the point of cmc, ΔH_{mic} is approximately 2.0 kJ/mol. Thus, we get $\Delta G_{\text{mic}} = -22.0$ kJ/mol and $\Delta S_{\text{mic}} = 80.5$ J/mol·K, which are consistent with those reported in the literature.⁹⁻¹⁰

The titration curve for SDS into MC deviates from the dilution curve at the first injection and the binding enthalpy starts to increase with increasing SDS concentration and decreases after reaching the maximum. As a result, a sharp endothermic peak appears. The critical aggregation concentration (cac) is shown as the inflection point in the leading edge of the endothermic peak according to the definition of Wang and Olofsson.¹¹ C_2 , the concentration where the titration curve in the polymer solution joins the dilution curve, is recognized at which the slope of the differential curve becomes zero. The calorimetric curve at 25 °C for addition of 200 mM SDS into 0.1 wt% MC solution gives $\text{cac} = 4.3$ mM and $C_2 = 14.2$ mM. The amount of surfactant bound to the polymer was nearly 9.9 mM calculated from the concentration range between cac and C_2 .

From a thermodynamic point of view, the enthalpy change at cac can be expressed as¹²

$$\begin{aligned} \Delta H = & \Delta H(\text{dilution of surfactant micelles and monomers}) + \\ & \Delta H(\text{demicellization of surfactant micelles}) + \\ & \Delta H(\text{binding of surfactant monomers to polymer chains}) \end{aligned} \quad (3-3)$$

Since the enthalpy changes of dilution and demicellization of SDS are experimentally observed to be small at 25 °C,¹³ the measured enthalpy change (denoted as ΔH_{obs}) is mainly attributed to the enthalpy change for the formation of polymer-SDS complexes, ΔH_{agg} . The Gibbs energy ΔG_{agg} can also be derived from:

$$\Delta G_{\text{agg}} = (1 + K)RT \ln(\text{cac}) \quad (3-4)$$

Since ΔH_{agg} is positive, the contribution to the Gibbs energy is dictated by $T\Delta S_{\text{agg}}$ as $\Delta G_{\text{agg}} = \Delta H_{\text{agg}} - T\Delta S_{\text{agg}}$. The endothermic peak corresponding to the cooperative binding describes the formation of SDS micelles on MC chains. When the cac is reached, the hydrophobic units of MC undergo a dehydration process for being solubilized by SDS micelles from water. Dehydration and formation of MC-SDS complexes are both entropy-driven and enthalpy-unfavorable because a large amount of heat, as represented by the sharp endothermic peak, is needed to disrupt the ordered water cages around the hydrophobic moieties of the polymer and SDS.

When the SDS concentration exceeds 5 mM, ΔH_{obs} decreases rapidly and intersects with the dilution curve at $C_{\text{SDS}} = 7.8$ mM, which is very close to the cmc of SDS in water. During the process, the number of SDS monomers per micelle increases and the growing electrostatic repulsion between the SDS head groups impedes the further binding of SDS to the polymer-SDS complex. The exothermic contribution to ΔH_{obs} should originate from the rehydration of MC segments that are expelled from the cores of the mixed micelles. This mechanism was first proposed by Wang and Olofsson^{11,14} for the EHEC-SDS system and subsequently promoted to the PEO-SDS system. Close to C_2 , the polymer chains intermingle in the SDS headgroups

region via ion-dipole association, which screens the contact between hydrophobic groups of surfactant and water, thus giving a favorable surface energy contribution. At C_2 , MC has been saturated with SDS and further addition of SDS tends to only dilute the free micelles.

Figure 3.6 Calorimetric titration curves for titration of 20 mM CTAB into MC solutions of different concentrations: 0.05 wt% (●), 0.1 wt% (■) and 0.25 wt% (▲) at 25 °C. (□) Dilution of concentrated CTAB (20 mM) in water at the same temperature.

3.3.2 Interaction between MC and CTAB

Calorimetric titration curves (solid symbols) for the addition of 20 mM CTAB into 0.05, 0.1 and 0.25 wt% MC solutions at 25 °C are shown in Figure 3.6, where the corresponding dilution curve of CTAB into water is presented for comparison. It is interesting to note that the initial dilution of CTAB into water shows a large enthalpy changes (~ 9.6 kJ/mol) while the enthalpy change for the dilution of SDS into water is very small (~ 1.3 kJ/mol). Here, the comparison between CTAB and SDS is made

under a reasonable condition that SDS and CTAB are diluted from their micellar solutions of 200 and 20 mM, respectively, which are 24 and about 22 times their individual cmc in water at 25 °C. The enthalpy of dilution for surfactant micelles is generally considered to be negligible as compared to the enthalpy of demicellization. Thus, the ΔH_{obs} plateau at CTAB concentrations < 0.9 mM is mainly attributed to the enthalpy of demicellization of CTAB into water. ΔH_{obs} decreases from 0.9 mM and then quickly reaches to the second plateau at high concentrations of CTAB. The second plateau indicates that the concentration of CTAB in titrate has been higher than its cmc and the titrant (20 mM CTAB solution) is only diluted and does not undergo demicellization anymore. Therefore, the second plateau can be used as a measure of the dilution enthalpy, ΔH_{dil} . The transition from the first plateau to the second one is related to the completion of CTAB demicellization. Thus, the inflection point (at the end of the first plateau) is the concentration of CTAB in the titrate, at which the CTAB micelles stop to demicellize. Therefore, the inflection point is the same as the cmc of CTAB. As indicated in Figure 3.6, the position of cmc is estimated to be around 0.94 mM, which is in good agreement with values reported in the literature.^{6,11}

Titration of CTAB into MC with different concentrations (0.05, 0.1, and 0.25 wt%) results in similar plateaus of ΔH_{obs} at low concentrations of CTAB. The ΔH_{obs} plateau increases in height with increasing MC concentration, indicating that the demicellization enthalpy of CTAB has been increased by the presence of MC in the titrate. This is based on an assumption that CTAB does not bind to MC chains when

its concentration is below the cac. The more MC in the titrate, the higher the first plateau. By analogy with the notation used in other polymer-surfactant systems,^{9,11} we regard the inflection point in each curve as the cac, the concentration at which the polymer-surfactant aggregation occurs. The cac, as indicated in Figure 3.6, decreases as the MC concentration increases: cac = 0.86, 0.76 and 0.62 mM for 0.05, 0.1 and 0.25 wt% MC respectively. The depression of cac by increasing MC concentration is a similar phenomenon that can be found in many nonionic polymer-ionic surfactant systems such as PPO-SDS,¹⁴ dextran-CTAC,¹⁵ and even hydrophobically modified polyelectrolyte DMRX-CTAC.¹⁶

After each cac, ΔH_{obs} gradually decreases with CTAB titration. For the titration of CTAB with the lowest concentration (0.05 wt %) of MC, the titration curve approaches the dilution curve at high concentrations of CTAB, meaning that the binding of CTAB to MC has been nearly saturated. In the concentration range from cac to saturation, it is believed that the demicellization of CTAB coexists with the binding of CTAB to MC. The decrease in ΔH_{obs} is because the amount of CTAB demicellization decreases with the titration. As MC is present in the titrate and CTAB binds to MC continuously, the binding saturation occurs only when all MC chains have been maximally bound by CTAB molecules.

The effect of MC concentration on the titration behavior can be understood easily. Before the cac, the ΔH_{obs} plateau increases with increasing MC concentration, which is attributed to the increased hydrophobicity of water by MC. After the cac, the ΔH_{obs} curve is higher at a higher concentration of MC. At a lower concentration of

MC, the binding saturation could be achieved by adding a smaller amount of CTAB. When the concentration of MC is higher, the amount of CTAB required for the binding saturation is larger. Thus, at a given CTAB concentration, the binding degree (defined as the number of CTAB molecules bound per chain of MC) decreases with increasing MC concentration. At the same time, the number of CTAB micelles that could demicellize increases with increasing MC concentration. As a result, a higher ΔH_{obs} is shown at a higher concentration of MC. The saturation concentration C_2 for 0.05 wt% MC should be close to 2.75 mM of CTAB, while C_2 for 0.1 and 0.25 wt% MC should be approximately 2 and 5 times that for 0.05 wt% MC respectively.

Figure 3.7 Calorimetric titration curve. (■) Addition of 100 mM SDS to 0.1 wt% CS solutions in 100 mM acetate buffer, pH 3.0, at 25 °C. (□) Dilution of SDS (100 mM) into the corresponding buffer solution.

3.3.3 Interaction between CS and SDS

Thongngam and McClements^{5,17} systematically studied the interactions of SDS

and CS using isothermal titration calorimetry. In the present work, we also carried out similar ITC experiments on this system and regarded it as a representative system for the interactions between cationic polyelectrolyte and anionic surfactant. In order to perform a comparable study, we choose room temperature 25 °C and pH 3.0 to examine the interaction of CS with SDS. A 100 mM SDS solution was isothermally titrated at 25 °C into either a buffer solution or a CS-containing buffer solution at pH 3.0. The ITC results are shown in Figure 3.7.

With the titration of SDS into the buffer solution at pH 3.0, a slow and endothermic increase in ΔH_{obs} is observed until about $C_{\text{SDS}} = 6.5$ mM. Then a mild decrease in ΔH_{obs} occurs from 6.5 mM and ΔH_{obs} tends to level off at high concentrations of SDS. The peak point around 6.5 mM is approximately regarded as the cmc of the surfactant in the pH 3.0 buffer solution and it is in close agreement with the value reported in the literature.⁵ At pH 3.0, CS is protonated to become a cationic polyelectrolyte that attracts anionic SDS electrostatically. This kind of ionic interaction is much stronger than hydrophobic association or ion-dipole attraction. Therefore, when SDS is titrated into CS at pH 3.0, a distinct exothermic valley is obtained in the concentration range from 0 to 5 mM, followed by an endothermic climb until about 11.5 mM at which the titration curve merges with the dilution one. The merging point, 11.5 mM, is defined as the saturation concentration C_2 . The broad endothermic plateau between the exothermic valley and C_2 , which is slightly positive in value, is considered to be due to some weak hydrophobic interaction between CS and SDS.

When SDS is titrated into a CS solution, the formation of either soluble or insoluble complexes between oppositely charged ions is an important concern. Thongngam and McClements⁵ used the turbidity measurements to study the formation of CS-SDS complexes, which was caused by the strong electrostatic attraction between CS (at pH 3.0, the initial concentration of CS was 0.1 wt%) and SDS. In the presence of NaCl, turbidity was found to reach a maximum at SDS = 6.7 mM, indicating the formation of CS-SDS complexes or aggregates. When those complexes are large in size, precipitation may occur. However, in an ITC experiment, the injection syringe-driven stirring ensures homogeneous mixing of SDS with CS at any time of titration. On the other hand, since turbidity did not decrease significantly beyond the maximum turbidity, the redissolution of those CS-SDS complexes at high concentrations of SDS still remained unknown.⁵

3.3.4 Interaction between CS and CTAB

Figure 3.8 shows ITC curves for the titration of 10 mM CTAB into a 0.1 wt% CS solution and a buffer solution, respectively, at pH 3.0 and 25 °C. Quite different from what we have observed in the oppositely charged polymer-surfactant system (for example, Figure 3.7), the enthalpy pattern observed in Figure 3.8 is similar to the interaction between MC and CTAB (Figure 3.6) which is a system of uncharged polymer-ionic surfactant.

Figure 3.8 Calorimetric titration curve. (■) Titration 10 mM CTAB into 0.1 wt% CS solution of pH 3.0 at 25 °C. (□) Dilution of concentrated CTAB (10mM) into pH 3.0 buffer solution at the same temperature.

As expected, the cmc of CTAB in the buffer solution decreased to 0.65 from 0.93 mM, which is attributed to the screening effect from the small amount of salt counterions in the buffer, since the electrostatic repulsion between surfactant headgroups normally opposes micellization.¹¹ Incorporation of positively charged CS into the buffer solution slightly shifts the titration curve upward. The cac is reflected as the inflection point of the enthalpic titration curve, which is 0.62 mM, slightly smaller than the cmc of CTAB. Compared with the above-discussed MC-CTAB system at the same 0.1 wt % polymer concentration, CS-CTAB experiences a smaller cac. This difference should be attributed to the lower hydrophobicity of CS than the MC. Furthermore, the positively charged CS may prevent CTAB monomers from aggregating by electrostatic repulsion, thus retarding the decrease in cac.

Bai et al.¹⁵ proposed two simultaneous processes during the aggregation of surfactant in hydrophobically modified polyelectrolytes with the same charge as the surfactant: a non-cooperative inclusion of surfactant molecules in the hydrophobic microdomains of polymer, which can be described as cic (critical incorporation concentration) and a cooperative self-aggregation of the bound surfactant, described by cac. The inflection point determined by ITC, which we call cac, is probably a combination of the two processes mentioned above. According to Bai et al.¹⁵, the cac values of all the *N*-(2-hydroxypropyl)-*N,N*-dimethyl-*N*-alkylammonium chloride-grafting dextran (DMRX)-CTAC/CTAB systems are lower than the cmc values of the corresponding free surfactants due to the presence of the polyelectrolyte backbone and pendant groups. They found that the higher the hydrophobicity of the polymer the lower the cac for the same hydrophobe concentration.

3.3.5 Interaction between KC and SDS

The interactions between a cationic polyelectrolyte and a cationic surfactant have been discussed in Section 3.3.4. The negatively charged counterparts are presented in this section. Calorimetric curves for titrating 200 mM SDS into 0.05, 0.1 and 0.25 wt% KC solutions at 25 °C are shown in Figure 3.10. The dilution curve (open squares) of SDS is also given.

Figure 3.9 Calorimetric titration curves for addition of 200 mM SDS to KC solutions of different concentrations: 0.05 wt% (●), 0.1 wt% (■) and 0.25 wt% (▲) at 25 °C. (□) Dilution of SDS (200 mM) in water at the corresponding temperature. The inset shows the plots at low SDS concentration.

The observed enthalpy curves exhibit the similar pattern as other polymer-surfactant systems with the same charges: alginate-SDS^{12, 14} and DMRX (D40Oct30)-DTAC.¹⁵ In the initial stage of the titration, ΔH_{obs} deviates from the dilution curve, depending on polymer concentration. This indicates that binding of SDS to KC takes place at low concentrations of SDS despite the existence of electrostatic repulsion. The cac is difficult to define, but it seems to be weakly dependent on polymer concentration: decreasing with increasing polymer concentration, which is in accordance with the results from D40Oct30-DTAC.¹⁵ The concentration, C_m , at which surfactant micelles start to form is referred to as the critical micelle concentration (cmc) in the presence of a polymer. As is well-known, polyelectrolytes have a similar effect on the cmc of

ionic surfactant as a simple salt does, but their effect is weaker than that of a salt.¹⁸ In this case, the increase of polyelectrolyte concentration enhances the strength of counterions (K^+ and Ca^{2+}) in the solutions, which in turn leads to a gradual decrease in C_m (inset in Figure 3.9). Moreover, the higher hydrophobicity of the titrate solution at a higher polymer concentration tends to promote micellization of the surfactant, leading to a smaller C_m .

At the beginning, the SDS micelles disassociate into free monomers. As both KC and SDS are anionic, ionic binding of SDS to KC is impossible. Thus, SDS can only bind to the hydrophobic groups or units of KC, which is an endothermic binding process where SDS tails bind to KC. Once the hydrophobic groups or units of KC are fully saturated by the SDS molecules, the free micelles appear at C_m . Beyond C_m , the overall enthalpy changes become smaller at a higher polymer concentration, indicating that the dilution of SDS to a polymer solution needs less heat than that for the dilution to water.

3.3.6 Interaction between KC and CTAB

As mentioned previously, a mixture of an ionic surfactant and an oppositely charged polyelectrolyte exhibits strong association dominated by electrostatic attraction between the ionized groups on the polyelectrolyte and the charged headgroups of the surfactant. The calorimetric curves for titrating 20 mM CTAB into 0.05 and 0.1 wt% KC solutions at 25 °C is shown in Figure 3.10 together with the corresponding CTAB dilution curve.

Figure 3.10 Calorimetric titration curves for titration 20 mM CTAB to 0.05 wt% (●) and 0.1 wt% (■) KC solutions at 25 °C. Open symbols denote dilution of CTAB (20 mM) in water at the corresponding temperature.

Electrostatic binding via Coulombic interaction occurs from the first point of titration and represented as a flat and broad exothermic plateau relative to the dilution curve of CTAB. For convenience, we call this the “lower plateau”. The small positive ΔH_{obs} values (around 2 kJ/mol) of the lower plateau could comprise two thermodynamically countervailing processes: demicellization of CTAB, which is highly endothermic ($\Delta H_{\text{demic}} = -8.2$ kJ/mol),¹¹ and the Columbic interaction between CTAB and KC which is highly exothermic. When $|\Delta H_{\text{demic}}| \geq |\Delta H_{\text{ele}}|$, where ΔH_{ele} is the enthalpy changes caused by electrostatic interaction between CTAB and KC, ΔH_{obs} will be above the horizon. A similar pattern of the calorimetric titration curve can be observed in the chitosan-sodium taurocholate (NaTC) interaction, which is another oppositely charged colloidal system.¹⁹⁻²⁰ Since ΔH_{mic} of SDS at 25 °C is very small as compared

to the electrostatic binding of SDS to CS, the exothermic valley below the horizon is observed in Figure 3.7 for the CS-SDS interactions. For the KC-CTAB interactions, the positive (i.e., endothermic) ΔH_{obs} means that the electrostatic interaction between KC and CTAB could not overwhelm the enthalpy changes of demicellization (ΔH_{demic}) because ΔH_{demic} of CTAB is quite large.

The effect of polymer concentration on the initial ΔH_{obs} is obvious: the lower plateau is longer for the higher concentration of KC. It is interesting to note that the length of the lower plateau at 0.05 wt% of KC is approximately half of that at 0.1 wt% of KC. Since the end of the lower plateau is considered as the finishing point of the electrostatic binding of CTAB to KC, more chains of KC in the solution need more CTAB molecules to bind electrostatically resulting in a longer plateau.

The lower plateau is followed by a steep increase at $C_{\text{CTAB}} = 0.5$ and 1 mM for 0.05 and 0.1 wt% KC respectively. At 0.1 wt% KC, after reaching a sharp peak, the enthalpy curve levels off from about 1.1 mM to form a pronounced endothermic plateau until about 2.2 mM. The endothermic plateau is attributed to the hydrophobic binding of CTAB to the polymer. In this range, the dissociation of CTAB micelles could take place to produce CTAB monomers that bind to the polymer. The hydrophobic binding of CTAB to KC takes place gradually and slows down slightly with increasing CTAB concentration. When the hydrophobic binding of CTAB to KC is nearly saturated, the enthalpy curve decreases dramatically and merges gradually with the dilution curve at high concentrations of CTAB.

Obviously, the endothermic plateau represents the whole process of the

hydrophobic binding of CTAB to KC until the saturation. The plateau height is approximately independent of the polymer concentration. The polymer chains in the 0.05 wt% solution are fully saturated by the bound CTAB molecules at about $C_2 = 2.1$ mM whereas those in the 0.1 wt% solution need more CTAB molecules to reach the saturation. Thus, C_2 for 0.1 wt% KC is expected to be about 4.2 mM.

It is well accepted that, in the presence of a polymer, polymer-induced micellization or demicellization may occur. When 20 mM CTAB is titrated into a KC solution, the demicellization of CTAB micelles is accelerated by the strong electrostatic attraction of KC. Once KC is electrostatically saturated by CTAB at the isopotential point (i.e., the end of the lower plateau), the additional surfactant micelles are diluted into the aqueous solution and then interact with the hydrophobic groups or units of KC. After the isopotential point, the further titration does not give the high ΔH_{obs} as the dilution does, implying that the additional CTAB micelles do not dissociate fully before reaching the cmc. It may be reasonably considered that in the presence of KC the CTAB micelles undergo only partial demicellization to produce surfactant monomers for hydrophobic binding to KC. The partial demicellization of CTAB and the hydrophobic binding of CTAB to KC result in the endothermic plateau of ΔH_{obs} . However, the polymer-induced cmc of CTAB is not present as a clear transition position on the calorimetric curve.

Similarly to the system of CS-SDS, the strong electrostatic attraction between KC and CTAB might lead to the formation of insoluble complexes. On the other hand, the insoluble complexes might be dissolved again by further adding CTAB. These

concerns have not been elucidated in the present work, but they should be verified through future work such as light scattering studies.

3.3.7 A general rule for polymer-surfactant interactions

When a micellar surfactant solution (i.e., $C \gg \text{cmc}$) is isothermally titrated into a dilute polymer aqueous solution, as already described in eq. (3-3), the thermodynamic process may generally include one, two or all three of following events: (i) dilution of surfactant micelles and monomers, (ii) demicellization of surfactant micelles, and (iii) binding of surfactant monomers or micelles to polymer chains, depending on the type of polymer-surfactant interactions and the stage of titration. Events i and ii are generally similar for all types of polymer-surfactant pairs. However, binding properties for a surfactant to a polymer or in other words polymer-surfactant interactions are greatly affected by the type of surfactant and the polymer.

Polymer-surfactant interactions are categorized into three types: hydrophobic interactions (van der Waals forces), ion-dipole interactions, and ionic interactions. If three types of interactions are possible at the same time, a general rule to determine the order (or priority) of surfactant binding to polymer would be ionic interaction (i.e., electrostatic attraction) > hydrophobic interaction > ion-dipole interaction. There are typically three cases to be taken into account. Case 1 is when an ionic surfactant is titrated into a non-ionic polymer solution; only hydrophobic interaction and ion-dipole interaction may be present. Case 2 is when an ionic surfactant is titrated into a solution of an ionic polymer with the same charge as the surfactant; the thermodynamic behavior will be similar to case 1. Case 3 is when an ionic surfactant

is titrated into a solution of an ionic polymer with the opposite charge as the surfactant; a strong polymer-surfactant interaction will be shown due to the strong electrostatic interaction between surfactant and polymer.

In this chapter, three types of polymers (MC, CS, and KC) and two types of surfactants (SDS and CTAB) were chosen to make six polymer-surfactant pairs which typically represent the above-mentioned three cases of polymer-surfactant interactions. Nonionic surfactants were not used in this study as they can not cover all the interactions. ITC results representing the typical polymer-surfactant interactions of the six pairs studied in this chapter are summarized in Figure 3.11. Calorimetric curves for titration of SDS into MC, CS, and KC are shown in Figure 3.11a, while those for the titration of CTAB into MC, CS, and KC are given in Figure 3.11b.

(a)

Figure 3.11 Combinative calorimetric titration curves: (a) addition SDS to MC (■), CS (●), and KC (▲); (b) addition of CTAB to MC (■), CS (●), and KC (▲) at 25 °C. The initial polymer concentrations were 0.1 wt%.

In Figure 3.11a, the MC-SDS system exhibits strong hydrophobic interaction as evidenced by the endothermic peak, followed by a weak ion-dipole interaction. KC-SDS does not show any ionic interaction as expected, but exhibits the weak hydrophobic interaction as compared with MC-SDS. Strong ionic interaction is found for CS-SDS as evidenced by a deep valley of exotherm, followed by the hydrophobic interaction between some hydrophobic moieties of CS and SDS tails. Finally, at high concentrations of SDS, any kind of polymer is fully saturated yielding in a similar ITC curve.

In Figure 3.11b, the titration of CTAB into MC gives a similar pattern of polymer-surfactant interaction as that for the titration into CS; this is expected

because no ionic interactions are possible in these two pairs of polymer-surfactant. In addition, the stronger hydrophobic interactions between MC and CTAB lead to the higher endothermic plateau than that for CS-CTAB. KC shows strong interaction with CTAB by largely reducing the high endothermic enthalpy changes caused by the demicellization of CTAB. After the isopotential point of KC is reached, the hydrophobic interaction with CTAB becomes possible.

3.4 Summary

The polymer-surfactant interactions for the six pairs of polymer-surfactant are systematically investigated using ITC. MC-SDS exhibited the strong hydrophobic interaction as evidenced by the endothermic peak, followed by a weak ion-dipole interaction. KC-SDS did not show any ionic interaction as expected, but exhibited weaker hydrophobic interaction than MC-SDS. The strong ionic interaction was found for CS-SDS, followed by the hydrophobic interaction between some hydrophobic moieties of CS and SDS tails. The titration of CTAB to MC resulted in the similar pattern of polymer-surfactant interaction to that for the titration of CTAB to CS. KC showed the strong interaction with CTAB by largely reducing the high endothermic enthalpy changes caused by the demicellization of CTAB. A general rule that determines the order (or priority) for a surfactant to bind to a polymer has been proposed based on our experimental results and discussion.

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Chapter 4

Influence of Cetyltrimethylammonium Bromide on Physicochemical Properties and Microstructures of Chitosan-Tripolyphosphate Nanoparticles

4.1 Introduction

Because chitosan (CS) is of biocompatibility, biodegradability and nontoxicity, it has attracted a great attention for pharmaceutical and biomedical applications. Uniquely, the glucosamine units on the CS backbone render this hydrophilic polymer a high density of positive charge in an acid solution. The strong electrostatic interactions between the amino groups of CS and proteins or genes make it a promising non-viral carrier candidate for drug delivery.¹⁻³ In particular, since small interfering RNAs (siRNA) have proven to be versatile agents for controlling gene expression in mammalian cells⁴⁻⁵, many research works have focused on the development of CS nanoparticles as a siRNA delivery system⁶⁻⁸. Katas et al. found that siRNA entrapped in CS nanoparticles formed by ionic gelation with tripolyphosphate (TPP) showed a good gene silencing effect.

The ionic gelation method for fabricating CS nanoparticles is simple and mild. Physicochemical properties of CS nanoparticles, acting as a drug carrier or a gene vector, have great effect on the delivery efficacy. Especially, particle size is one of the most significant factors which determine the efficiency of nanoparticle's uptake in mucosal and epithelial tissue and the intracellular trafficking of the particles.⁹⁻¹⁰ For

example, Desai et al. reported that the particle uptake by caco-2 cells depends significantly upon the particle's diameter. The 100 nm diameter particles have 2.5-fold higher uptakes on a weight basis than the 1 μ m diameter particles and 6-fold higher than the 10 μ m diameter particles.¹¹ Accordingly, the success in the development of CS nanoparticles useful for drug or gene delivery is determined by a full understanding of the relations between the physicochemical properties of the nanoparticles and a given biological environment.

Studies on the effect of preparation condition on nanoparticle's physicochemical properties underwent a fast growth in recent years. Grenha et al. found that a higher concentration solution of CS could lead to larger size particles.³ Gan et al. reported that variations in molecular weight of CS, CS concentration, CS to TPP ratio and pH could affect the physicochemical properties such as particle size, intensity of surface charge, and tendency of particle aggregation.¹² Thus, speedy fabrication of the CS nanoparticles can be achieved with predetermined properties. López-León et al. analyzed the electrokinetic properties and colloidal stability of CS nanoparticles as a function of pH and ionic strength of the medium.¹³ They observed that CS-TPP particles showed the volume phase transitions (swelling-shrinking) when pH of the immersing medium was changed from 4 to 7. Tang et al. proposed a method to manipulate the particle size using ultrasonication.¹⁴ Ultrasonication was found to cause the decrease in the mean diameter and in polydispersity of the CS-TPP nanoparticles. Tsai et al. used another method, blade shearing, to reduce the particle size by degrading the CS molecules.¹⁵

Besides the above mentioned methods for modulation of CS-TPP nanoparticles, surfactants, as a special colloidal and interfacial modifier, have high potential applications in fabrication of CS nanoparticles. Calvo and co-workers reported that size and zeta potential of CS-TPP nanoparticles could be conveniently modulated by varying the ratio of CS to poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) diblock copolymer.¹⁶ Thongngam et al. investigated the interaction between CS and sodium dodecyl sulfate (SDS), a typical anionic surfactant, via isothermal titration calorimetry (ITC) and surfactant-selective electrode (SSE).¹⁷⁻¹⁸ They found that SDS-CS binding was dominantly affected by the electrostatic interactions between CS and SDS, and the NaCl present had a little influence on the amount of SDS bound to polyelectrolyte at saturation. However, studies on the interaction between CS and cationic surfactants such as cetyltrimethylammonium bromide (CTAB) are rare in the literature. Li and co-workers recently reported the aggregation behavior of carboxymethylchitosan (CMCS) in the presence of CTAB.¹⁹ However, no reports have been found on the effect of CTAB on modulation of CS-TPP nanoparticles. Thus, the aim of this study is to investigate the influence of CTAB on the physicochemical and structural properties of CS-TPP nanoparticles.

It is also worthy to note that a great deal of attention has been paid to the structural and dynamical studies of CS in aqueous solution by means of laser light scattering (LLS).²⁰⁻²³ Particularly, the combination of static and dynamic light scattering techniques provided a powerful tool for understanding of the structural and dynamical properties of a semi-rigid polyelectrolyte such as CS, in aqueous solution.

However, few papers have reported the characterization of CS-TPP nanoparticles by LLS in detail. Therefore, another objective of this study is to explore the mechanisms for the ionic gelation of CS with TPP and the effect of CTAB on the formation of CS nanoparticles with the aid of LLS. In addition, zeta potential and transmission electron microscopy (TEM) have been used to obtain the supportive information on the effect of CTAB on the physicochemical properties and microstructures of CS-TPP nanoparticles.

4.2 Experimental

4.2.1 Materials

Medium molecular weight (MMW) chitosan (CS) with a nominal degree of deacetylation of 75–85%, was purchased from Sigma-Aldrich. Pentasodium triphosphate (TPP) and cetyltrimethylammonium bromide (CTAB) were also obtained from Sigma and used as received. Other reagents, such as acetic acid (HAc) and sodium hydroxide (NaOH), are of analytical grade. Deionized water was obtained from a Milli-Q[®] filter unit (Millipore, USA).

4.2.2 Purification of CS

Since the commercial CS material in the form of fibril flakes contained some insoluble impurities. A modified purification method according to Gan et al.¹² was employed, and details were described in Section 3.1.1.1.

4.2.3 Preparation of CS-TPP nanoparticles

Four different concentrations of CS solutions (0.5, 1.0, 1.5 and 2.0 mg/mL respectively) were prepared by dissolving the purified CS into acetic acid solutions. In order to examine the electrolyte effects on the physicochemical properties of the CS nanoparticles, two kinds of acetic acid solutions, which contained 0.2 M HAc/0.1 M NaAc and 0.2M HAc respectively, were used in this chapter. For convenience, the former acetic acid solution is expressed as “in salt” and the latter as “no salt” in this report. All the solutions of CS obtained were clear and transparent. The concentration of TPP was fixed at 1.0 mg/mL, and CTAB was dissolved in deionized water at 40 °C with a concentration of 5.0 mg/mL.

Based on the optimum ionic gelation conditions discussed by Calvo et al.¹⁶, CS nanoparticles were produced by dropwise addition of the TPP solution into a CS solution (0.45 µm filtered) with a selective CS to TPP ratio (in weight) of 5:1 (e.g. 10 mL of 1.0 mg/mL CS solution was mixed with 2 mL of 1.0 mg/mL TPP solution). The gelation process was carried out under constant magnetic stirring at room temperature (22 °C). The nanoparticles were then incubated at room temperature for 30 min before use or further analysis. To evaluate the surfactant's effects on the nanoparticles, CTAB was premixed with the CS solutions at a constant volume fraction (1:25) of the final solutions (e.g. 0.5 mL CTAB addition to 10 mL of 1.0 mg/mL CS and 2 mL TPP system). The ionic gelation process of the CTAB-containing CS solutions with TPP is similar with the one described above.

4.2.4 Physicochemical characterization of CS-TPP nanoparticles

4.2.4.1 Laser light scattering (LLS)

Static light scattering (SLS) and dynamic light scattering (DLS) experiments were performed by means of a commercial light scattering spectrometer (Brookhaven BI-200M) equipped with an argon ion laser operating at $\lambda = 488$ nm and a BI9000AT multi- τ digital time-correlator. For SLS, the instrument was calibrated with toluene to ensure that the scattering intensity from toluene had no angular dependence in the testing angular range. For DLS, 90° measurement angle and 3 min correlation measurement time were used as standard parameters, and photon kilo-count rate per second (kcps) was controlled below 1000 by adjusting light filters. All the particle suspension samples were centrifuged at 10000 rpm for 10 min to remove dusts before light scattering testing. The measurements of particle size were carried out at 25 ± 0.1 °C in triplicate.

For SLS, the angular dependence of the excess absolute time-averaged scattered intensity, known as the excess Rayleigh ratio, R_θ , of a dilute polymer solution can be measured at different concentrations, c (g/mL), and scattering angles θ . R_θ is related to the weight-average molecular weight, M_w , through the Zimm equation:

$$\frac{Kc}{R_\theta} \cong \frac{1}{M_w} \left(1 + \frac{1}{3} \langle R_g^2 \rangle q^2 \right) + 2A_2c \quad (4-1)$$

where $K = 4\pi^2 n^2 (dn/dc)^2 / (N_A \lambda_0^4)$ and $q = (4\pi / \lambda_0) \sin(\theta/2)$ with N_A , dn/dc , n and λ_0 being the Avogadro number, the specific refractive index increment, the

solvent refractive index and the wavelength of light *in vacuo*, respectively. A_2 is the second virial coefficient and $\langle R_g^2 \rangle_z^{1/2}$, or simply R_g , the root mean square z-average radius of gyration of the polymer chain in solution. At a constant concentration, eq. (5-1) can be rewritten as the partial Zimm or Berry equation:

$$\left[\frac{Kc}{R_\theta} \right]_{c \rightarrow 0} = \frac{1}{M_w} \left(1 + \frac{1}{3} \langle R_g^2 \rangle q^2 \right) \quad (4-2)$$

R_g and M_w can be determined by plotting Kc / R_θ or $(1/I)^{1/2}$ against q^2 .

In DLS, the decay rate Γ is the inverse of the relaxation time τ , while the scattering vector q dependence on the Γ can be used to determine the physics of the decay mode in the decay time distribution function. In dilute solution, the slopes of Γ versus q^2 are related to the translational diffusion coefficients D of particles in solution according to Equation 4-3.

$$\Gamma = Dq^2 \quad (4-3)$$

Based on the Stokes-Einstein relationship, the apparent hydrodynamic radius R_h can be obtained from DLS measurements,

$$R_h = \frac{kT}{6\pi\eta D} \quad (4-4)$$

where k is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity.

4.2.4.2 Zeta potential

A zeta potential analyzer (ZetaPALS, Brookhaven, USA) was used to determine the electrophoretic mobility (EPM) of charged CS particles in solution and to calculate the zeta potential. The relationship between mobility and zeta potential

can be shown as,

$$\mu = \xi \frac{\varepsilon}{\eta} f(ka) \quad (4-5)$$

where ε is dielectric constant, η is the solvent viscosity, and $f(ka) = 1.5$ for Smoluchowski limits. The measurements were performed at 25 ± 0.1 °C under the Smoluchowski approximation,²⁵ and 3 runs of 20 cycles were chosen for a good reproducibility. The instrument automatically optimizes the signal intensity within the range of 200~300 kcps to keep the ratio of sample to reference count rates to be a constant approximately.

4.2.4.3 Transmission electron microscopy (TEM)

The morphological properties of the CS-TPP nanoparticle were characterized using TEM (JEM-2010, JEOL, Japan) operated at an accelerating voltage of 160 kV. The TEM samples were prepared as follows. The nanoparticle suspension was dripped onto a 400-mesh copper grid pre-coated with Formvar and stained by 2 wt% phosphotungstic acid.²⁶ The sample was air-dried at room temperature for more than 4 hours before viewing on the TEM.

4.3 Results and discussion

In an acetic acid solution, CS is water-soluble due to the protonation of amino groups and it exhibits a polyelectrolyte character. The electrostatic repulsion between charges along a CS chain will affect the local flexibility of the chain so that the chain will tend to adopt extended conformation.²² When CS and TPP are mixed with each other to form gel complexes, the initial state of CS in the acetic acid solution has a

great effect on the physicochemical properties of a final product. In a 0.2 M HAc solution, the degree of protonation of CS can be regarded as approximately 100 % covering all the polyelectrolyte concentrations we used.²⁷ Considering the CS-TPP suspension as a metastable system, changing the ionic strength and pH value can dramatically alter the aggregation behavior of CS molecules.^{13,25} In the present work, the effects of CTAB on the CS-TPP nanoparticles are studied by varying the CS concentration, solution salinity and the concentration of itself.

Figure 4.1 Dependence of CS-TPP particle sizes on concentration of CS formed under four solution conditions: (i) salt-containing and CTAB-free; (ii) salt-containing and CTAB-containing; (iii) salt-free and CTAB-free; and (iv) salt-free and CTAB-containing. The initial concentrations of salt and CTAB were 0.1 M and 5 mg/mL respectively.

4.3.1 Effect of CS concentration

CS can be immediately formed into gel-like particles upon contacting with polyanions like TPP. The positively charged amino groups along the polymer chains bind with the negatively charged phosphates of TPP by electrostatic attraction. Figure

4.1 shows the CS-TPP particle size D_h over a concentration range of CS from 0.5 to 2.0 mg/mL, where the mass ratio of CS to TPP was fixed at 5:1. It is demonstrated that the nanoparticles formed had a general tendency to increase the average hydrodynamic diameter with increasing CS concentration both in the presence and the absence of salt and CTAB. This result is in accordance with and enriches the reported work on modulation of CS-TPP nanoparticles^{12,16}. It is easy to imagine that in a higher concentration solution there are more CS chains per volume to result in the formation of larger particles upon adding a crosslinking agent. The combination of static and dynamic light scattering techniques further testified this speculation.

Two kinds of particle radius, R_h and R_g , can be measured by DLS and SLS respectively. According to classical polymer physics, R_h represents the radius of an equivalent hard sphere with the same diffusion coefficient; and $R_g = \langle R_g^2 \rangle_z^{1/2}$, $\langle R_g^2 \rangle$ is defined as the average square distance between monomers in a given conformation and the centre of polymer mass²⁸. The parameter $\rho (= R_g / R_h)$ can be used to examine the morphological microstructures of aggregates, micelles, or gel networks. In a good solvent, polymer chains can be regarded as Gaussian chains, and R_h is smaller than R_g . Theoretically, ρ is 1.5 for Gaussian chains. When the polymer chains collapse into spheres, R_h approaches closely the radius of a real solid sphere. In this case, R_h is larger than R_g , giving $\rho = 0.77$.²⁸⁻³⁰

The relaxation time distribution functions for CS-TPP nanoparticles in the salt solutions of different CS concentrations were plotted in Figure 4.2. Each of the distribution functions is unimodal, and the peak shifts to the right (i.e. longer

relaxation time) with increasing concentration of CS, implying that the particle size increased with CS concentration. The root of the inverse of the scattering intensity, $(1/I)^{1/2}$, was plotted in Figure 4.3 as a function of q^2 . According to eq. (4-2), R_g could be determined from the slope and the intercept.

Figure 4.2 Characteristic relaxation time distribution functions obtained by DLS at 90° for CS-TPP nanoparticles formed at different CS concentrations from 0.5 to 2.0 mg/mL in salt-containing solutions without CTAB.

Figure 4.3 Typical Berry plots obtained from SLS for CS-TPP nanoparticles formed at different CS concentrations from 0.5 to 2.0 mg/mL in salt-containing solutions without CTAB.

Figure 4.4 Effect of CS concentration on R_g/R_h values of CS-TPP nanoparticles formed under all the conditions used in this study. The initial concentration of CTAB was 5 mg/mL.

The ρ values for the nanoparticles formed in all the solutions with and without salt (0.1 M NaAc) and CTAB (5 mg/mL initial concentration) are given in Figure 4.4. From Figure 4.4, it is known that the value of ρ decreased approximately from 0.9 to 0.5 with increasing CS concentration. The nanoparticles formed at 0.5 mg/mL of CS could not be regarded as hard spheres because its ρ values (> 0.90) are larger than 0.77. Interestingly, Ravi et al.³⁰ suggested a core-shell structure for poly(methacrylic acid)-*block*-poly(methyl methacrylate) in aqueous medium which had ρ near to 0.9, and they proposed a loosely extended microstructure. Thereby, we could imagine the nanoparticles formed at 0.5 mg/mL as fluffy colloidal complexes. The ρ values for the nanoparticles formed at 1.0 mg/mL are near 0.80, which are quite close to 0.77. This indicates that these nanoparticles were so compact that they could be approximately termed as hard spheres. At the high concentrations (1.5 and 2.0

mg/mL), the values of ρ are below 0.77 and ρ decreased with increasing concentration of CS. This tendency suggests that the higher the CS concentration, the more compact the nanoparticles. As for the ρ values below 0.77, this deviation from the theoretical prediction signifies a non-uniform distribution of polymer segments within the nanoparticles as the similar results could be easily found in the literature.²⁴

All of these solution conditions resulted in the similar variation of R_g / R_h with increasing CS concentration. An approximately linear relation could be deduced: $R_g / R_h = kc + d$, where k and d are the constants and c represents the CS concentration in mg/mL. Thus, it is concluded that the crosslinking density and/or the degree of compaction of the nanoparticles in the aqueous solutions were primarily determined by the concentration of CS while the presence of CTAB or salt only had a minor effect on R_g / R_h as discussed later.

4.3.2 Effect of salt addition

As shown in Figure 4.1, the salt addition has a tendency to increase the particle size of CS, especially at high CS concentrations. For example, at 2 mg/mL of CS and in the absence of CTAB, the average particle size was increased from about 290.1 nm in the salt-free solution to 366.8 nm in the salt-solution. This effect can be explained by the enhanced hydrophobicity of CS by the salt, which is a typical “salt-out” effect.³¹ When CTAB was present, we could still observe the salt effect on the particle size, but it was weaker because CTAB had the opposite effect to decrease the particle size as discussed in section 5.3.3.

Figure 4.5 Effect of CTAB on zeta potential of CS-TPP particles in salt-containing and salt-free solutions at different concentrations of CS. The initial concentration of CTAB was 5 mg/mL.

The effect of the salt on the zeta potential is significant. The salt-free solutions (with and without CTAB) exhibited high zeta potentials from 45.6 to 72.3 mV while the zeta potentials of the salt solutions dropped to a level below 35 mV, as shown in Figure 4.5. The salt effect on the decrease in zeta potential is attributed to the screening effect that the salt ions covered the cations of CS from exposing.³²

In the present work, the salt type and salt concentration were fixed as NaAc and 0.1 M respectively, and we did not study the effect of salt type and salt concentration on the formation of CS nanoparticles. This is because our main objective was to study the effects of CTAB. The effects of CTAB in the presence and the absence of the salt were different and we discuss them in the next section.

4.3.3 Effects of CTAB

The effect of CTAB is considered to be a combination of two opposite factors.

On the one hand, CTAB dispersed on the surface of CS nanoparticles tends to increase the inter-particle electrostatic repulsion force, thus making the gel networks more compact. On the other hand, CTAB encapsulated in the nanoparticles has a tendency to cause the networks to swell by the intra-particle electrostatic repulsion force. The balance between inter- and intra-particle electrostatic interactions could offset the effect of CTAB on the ratio R_g / R_h .

In the salt solution, at a given concentration of CS, CTAB was found to always reduce the sizes of nanoparticle in salines (Figure 4.1). For example, at 2.0 mg/mL of CS, the particle diameter was reduced from 366.8 to 320.8 nm by adding 5 mg/mL CTAB. This result is similar with the effect of CS molecular weight on the particle size.¹² The mechanism for CTAB to reduce the sizes of CS-TPP nanoparticles can be explained through the relationship between gel particle stability and zeta potential. Figure 4.5 shows the zeta potentials of CS-TPP nanoparticles formed in different solutions. In the presence of the salt, the ionic shielding function tends to reduce the particles' mobility, thus diminishing their zeta potential.³² As observed in Figure 4.5, before adding CATB, the zeta potentials of the particles in the salt solutions were approximately 27 mV, which were quite lower than those of the particles formed in the salt-free solutions. In the salt solutions, the addition of CTAB increased the zeta potentials of the particles only by 4–5mV, reaching to a level of 31–32 mV. On the other hand, in the salt-free solutions, CTAB was able to increase the zeta potential by 6 to 20 mV, which was greater than that in the salt-containing solutions.

Now we can explain the effect of CTAB on the decrease in the particle size.

As CTAB is a cationic surfactant and CS is a cationic polyelectrolyte, electrostatic interaction between CTAB and CS is impossible. However, CTAB is able to bind to CS chains through weak hydrophobic interaction.³³ The hydrophobic binding of CTAB results in the increase in the cationic charge density as per volume of CS-TPP nanoparticles (assuming that the density of CS-TPP nanoparticles is not changed by adding CTAB). Thus, the decrease in the average sizes of CS particles could be caused mainly by the increased electrostatic repulsion between CS particles when CTAB was present. At the same time, we may also consider that the probability of particle aggregation was lower when the surface charge was increased by adding CTAB, thus resulting in the formation of smaller particle sizes. Due to the presence of salt ions, the effect of CTAB on the zeta potential was not as strong as that in a salt-free solution of CS.

While the effects of salt and CTAB on zeta potential are significant, the effect of CS concentration is not notable especially when the salt was present. As shown in Figure 4.1, the particle size increases with CS concentration significantly but the zeta potential does not increase with CS concentration, indicating that the surface charge density of CS particles is approximately constant and independent of particle size. Here, the empirical rule of ± 30 mV being the borderline of colloidal stability is considered to be valid for our CS-TPP system.³⁴

In the salt-free solutions without adding CTAB, Figure 4.1 demonstrates the following facts: there is a negligible difference in particle size formed with and without NaAc when the CS concentration was lower (0.5 and 1.0 mg/mL), but the

difference became significantly large when the concentration of CS was higher (1.5 and 2.0 mg/mL). However, the addition of CTAB had an inconspicuous and inconsistent effect on particle size according to the variation of CS concentration. It is speculated that there exists a critical overlapping CS concentration c^* near 1.0 mg/mL between dilute and semi-dilute regimes. Using intrinsic viscosity measurement, we got the $c^* = 0.63\text{--}1.1$ mg/mL. This value is consistent with that found by E. Buhler et al.'s where c^* ranged from 0.55 to 1.0 mg/mL at different salt concentrations as determined using light scattering methods²¹. In the dilute regime (below c^*), the typical good solvent behavior was shown and semi-rigid CS had an extended chain conformation and less inter-molecular reaction due to the longer molecular distance. The similar phenomenon could be deduced when CS is crosslinked. Simply speaking, the inter-molecular electrostatic interaction at low concentrations of CS had a weak effect on the microstructure of nanoparticles than that at high concentrations of CS. This explains why the nanoparticles' zeta potential (formed at 0.5 mg/mL CS) increased from 26.5 mV to 51.3 mV (Figure 4.5) while the nanoparticle size remained almost unchanged. By contrast, nanoparticles formed at 2.0 mg/mL show a sharp decrease in particle size from 366.8 nm to 290.1 nm while the zeta potential increased from 27.2 mV to 45.6 mV. Because of the smaller spatial distance between polymer chains at high concentrations, the average size of CS-TPP particles became more sensitive to the mutual repulsion forces. In the semi-dilute regime, the strength of electrostatic repulsion force determines the average number of polymer chains involved in a single nanoparticle. Therefore, in the salt solutions, the

screened repulsion forces were so weak that more crosslinked CS spherules tend to aggregate together to yield large particles.

In the presence of CTAB, CTAB exhibits relatively weak effect on the particle size in the salt-free solutions according to Figure 4.1. The high density of surface charge (i.e. zeta potentials of above 50 mV) in the salt-free solutions (as known from Figure 4.5) allowed the nanoparticles to prevent agglomeration effectively. Since the zeta potentials were much higher than the empirical borderline of 30 mV to maintain the particle morphology stable in the aqueous medium, the addition of CTAB had the limited contribution to further reduce the average hydrodynamic diameter of the particles. In fact, the addition of CTAB even had an adverse effect on reducing the particle size when the CS concentration was 2.0 mg/mL.

A possible interpretation is proposed as follows. Firstly, the CTAB molecules and CS chains dispersed uniformly in the solution where there were Columbic repulsion, hydrophobic interaction and hydrogen bond among them. When TPP was added, the gel particles were formed, and the positively charged CTAB molecules would be squeezed out from the crosslinked networks of CS by the high internal charge intensity. Some CTAB molecules would successfully escape and “anchor” onto the surface of gel particles or move into the solution to exist as monomers. The fraction of CTAB molecules in the nanoparticles is expected to be larger when the concentration of CS is higher because the more compact and larger networks were formed (this has been discussed in Section 4.3.1). The gel particles tend to swell by the electrostatic repulsion between the encapsulated CTAB’s headgroups and the

protonated amino groups. This is why the particle size increased with addition of CTAB when the CS concentration was high. Of course, this phenomenon should also occur in the salt solutions, but the zeta potential increment by adding CTAB led to a predominant role in reducing the particles size, which countervailed the expanding effect of the enveloped CTAB.

Although CTAB had the very limited effect on modulation of particle size in the salt-free solutions, it still increased the zeta potential remarkably, which could facilitate adhesion and transport properties of the nanoparticles for anionic biomolecules such as proteins, DNA or RNA.³⁵ Additionally, the loading and transfection efficacy of nanoparticles as carriers is expected to increase.³⁶

In order to further understand the role of CTAB in the formation of CS-TPP nanoparticles (in semi-dilute CS solutions), we investigated the variations in the particle size and zeta potential with changing CTAB concentration as shown in Table 5.1. Both in the salt-free and the salt-containing solutions, it appears that a minimum hydrodynamic diameter exists at 10 mg/mL of CTAB. Herein, the TEM micrographs have been obtained, which provide us with the supportive information for understanding the role of CTAB.

Table 4.1 Variations of CS-TPP particle size and zeta potential with adding different concentrations of CTAB into 1.5 and 2.0 mg/mL CS solutions respectively

<i>CTAB initial</i>		<i>Particle size D_h (nm)</i>		<i>Zeta potential (mV)</i>	
<i>concentration (mg/mL)</i>		1.5 mg/mL CS	2.0 mg/mL CS	1.5 mg/mL CS	2.0 mg/mL CS
In salt solutions	0	277.4±4.1	366.8±10.4	27.2±0.4	27.2±1.4
	5	258.4±3.6	320.8±4.3	30.7±0.81	32.9±0.4
	10	230.8±2.7	307.0±2.9	31.3±1.0	34.7±0.4
	20	237.9±4.9	328.7±13.7	33.7±0.6	36.6±0.4
No salt solutions	0	230.0±6.2	290.1±8.7	53.0±0.9	45.6±2.3
	5	231.3±1.8	314.1±3.2	61.2±0.8	51.3±0.9
	10	204.2±1.2	286.6±1.5	51.1±1.1	53.1±0.7
	20	214.4±4.4	346.3±5.4	49.5±1.2	50.8±0.5

The critical micelle concentration (cmc) of CTAB in water was reported to be 0.92 mM at 25 °C.³⁷ The initial concentrations (i.e. 5, 10 and 20 mg/mL) of CTAB used in this study covered the concentration range for CTAB to form monomers to micelles. For example, 10 mg/mL (or 1.1 mM in final solution) is slightly higher than but very close to the cmc, while 5 and 20 mg/mL are below and above the cmc respectively. In the presence of salt and polyelectrolyte, cmc of CTAB will shift. In general, when more other solutes are dissolved in water, cmc of a surfactant will become smaller because less water molecules are available for the surfactant molecules or in other words the water is more hydrophobic than before adding the solutes. Typically, when a salt or a relatively hydrophobic polymer is added, cmc of the surfactant will decrease remarkably.^{18,37}

Figure 4.6 TEM microphotographs of CS-TPP nanoparticles formed in 2.0 mg/mL CS salt-containing solutions with incorporation of different concentrations of CTAB: (a) 0; (b) 5; (c) 10; (d) 20 mg/mL.

The TEM micrographs shown in Figure 4.6 are interesting and significant. In the CTAB-free solution (Figure 4.6a), the CS particles tend to agglomerate due to the weak inter-particle repulsion. When 5 mg/mL CTAB was added (Figure 4.6b), the inter-particle repulsion was increased by CTAB, preventing the particles from agglomeration. When the concentration of CTAB is further increased (Figure 4.6c) to be equal to or higher than the cmc, the micelles are formed. These micelles include the mixed micelles formed from CTAB molecules and chain segments of CS. As the mixed micelles present mainly on the surface of nanoparticles impart stronger inter-particle repulsion, the bigger particles could collapse into smaller particles in order to

minimize the surface tension. Thus, the smaller particles were obtained as observed in Figure 4.6c. In Figure 4.6c, the small and white congeries surrounding the black particles of CS should be attributed to the aggregated CTAB micelles. At the highest concentration of CTAB, the bigger particles were formed (Figure 4.6d).

4.3.4 A proposed mechanism

Scheme 4.1 A schematic illustration of the proposed microstructures of the nanoparticles formed when adding CTAB of different initial concentrations (low to high from left to right).

Scheme 4.1 shows a schematic description of the microstructures of CS nanoparticles formed in different concentration regimes of CTAB, which were speculated in terms of the TEM images in Figure 4.6 and the work by Kjøniksen et al.³⁸⁻³⁹. The polymer concentrations for the microstructures proposed in Scheme 4.1 should be within the semi-dilute range and well above the overlapping concentration

c^* .

When CTAB of different initial concentrations (from low to high) is added to a CS solution of a given concentration ($C > c^*$), three cases can be classified in terms of CTAB concentration: $C_{CTAB} < cmc$, $C_{CTAB} \sim cmc$, and $C_{CTAB} > cmc$. When $C_{CTAB} < cmc$, CS chains bound by CTAB molecules aggregate to form CS nanoparticles. The bound CTAB may increase the inter-particle repulsion to prevent agglomeration of the nanoparticles, resulting in smaller particle sizes. When C_{CTAB} is about equal to the cmc , the CTAB micelles are formed, which are possibly mixed with CS segments. The mixed micelles are unlikely present inside the nanoparticles due to electrostatic repulsion. They likely reside on the surface of nanoparticles. As a result, the inter-particle repulsion becomes stronger, which causes bigger particles to collapse into smaller particles. When $C_{CTAB} > cmc$, free micelles of CTAB can be formed. At high concentrations of CTAB, excess CTAB molecules could be encapsulated by the particles and then reside inside the particles as bound molecules or mixed micelles. These interior CTAB micelles could increase the internal electrostatic repulsion to result in some expanded particles.

4.4 Summary

Nanoparticles of CS were prepared using TPP as a crosslinking agent and the influences of surfactant CTAB on the physicochemical properties of the CS-TPP nanoparticles were studied by LLS, zeta potential, and TEM. The addition of CTAB could reduce the hydrodynamic diameter of nanoparticles effectively in the salt solutions and simultaneously increase the zeta potential of the nanoparticles. The

combination of DLS and SLS methods was firstly applied to study the CS-TPP nanoparticles. It was found that the ratio $\rho (=R_g / R_h)$ decreased approximately from 0.9 to 0.5 with increasing CS concentration, and the salt and CTAB did not affect the ratio ρ significantly. The effect of CTAB concentration on the formation of CS-TPP nanoparticles was also studied. A critical overlap concentration c^* of CS around 1.0 mg/mL was used to explain the inconspicuous changes in particle size. In a semi-dilute solution of CS, the minimum particle size could be reached near the cmc of CTAB. The mechanism behind the phenomenon has been proposed and discussed.

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Chapter 5

Comb-Like and Stimuli-Responsive Graft Chitosan Copolymer Synthesized Using ATRP and Active Ester Conjugation Methods

5.1 Introduction

Grafting of chitosan (CS) is a useful way to improve properties of CS such as increasing chelating or complexation properties¹⁻², bacteriostatic effect³ or enhancing adsorption properties⁴. Several initiator systems such as, ammonium persulfate (APS), potassium persulfate (PPS), ceric ammonium nitrate (CAN), 2, 2'-azobisisobutyronitrile (AIBN) and ferrous ammonium sulfate (FAS) have been used and well summarized by Jayakumar and coworkers.⁵ Grafting of synthetic polymers onto CS will introduce desired properties and broaden their potential applications, while maintaining CS intrinsic biocompatibility.⁶ However, due to its conventional nature, free radical polymerization is unable to control molecular weights of grafted chain or minimize chain transfer of radicals from the growing grafted chain end, leading to chain scission. Radical polymerization methods exhibiting controlled/"living" character have developed very fast in recent years. They are capable of not only generating specific macro-radicals, but also yielding narrow molecular weight distributions. In 1995, Matyjaszewski⁷ and Sawamoto⁸ independently developed the transition metal catalyzed living radical polymerization, known as atom transfer radical polymerization (ATRP). ATRP seems to be the most

robust method to control radical polymerization of various vinyl monomers.⁹⁻¹⁰

Graft copolymerization of vinyl monomers onto CS and other natural polymers with controlled architecture using ATRP has recently attracted much interest. Most of the modifications have been performed using “grafting from” methods. Tahlawy et al.¹¹ prepared CS macroinitiator by the reaction of CS with 2-bromoisobutylryl bromide (2-BIB), and then methoxy capped (PEG 350) methacrylate was grafted from it. Li et al.¹² modified CS beads by grafting polyacrylamide via surface-initiated ATRP and found that the enhanced and selective removal of mercury ions was achieved. Similarly, Liu and Su¹³ successfully grafted polystyrene onto CS particles using heterogeneously surface-initiated ATRP. Other kinds of polysaccharides, such as cellulose, pullulan, dextran and acetylated starch were also modified via ATRP.¹⁴⁻¹⁶ However, due to the poor solubility of polysaccharides in traditional ATRP solvents, most of reported polymerizations took place heterogeneously and resulted in low degree of substitution (DS).

A “grafting onto” approach is more promising under such a background. Utilizing a carboxyl activating agent, 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide) (EDC), for amide bonding with primary amines, several water-soluble CS graft copolymers were synthesized.¹⁷⁻¹⁹ Meanwhile, Yang’s group prepared PEG-*g*-CS using purchased PEG-nitrophenol carbonate and succinimidyl ester of PEG propionic acid (mPEG-SPA) respectively, and its gene delivery efficiency as a non-viral carrier was evaluated.²⁰⁻²¹ Since the active esters can react readily with a variety of nucleophiles such as primary and secondary amines and these reactions progress

almost quantitatively in most cases, this technique is widely used for conjugation of proteins and functionalized solid surfaces.²²⁻²⁴ Another advantage of using an active ester is that one precursor can lead to many functional polymers varying in chemical functionality.²⁵⁻²⁶

Poly[(2-dimethylamino)ethyl methacrylate] (PDMAEMA) was chosen as the side chains due to its excellent biocompatibility, hydrophilicity, pH- and thermo-sensitivities.²⁷⁻²⁹ To date, in the literature, the reported grafting of DMAEMA onto CS were mainly based on conventional free radical and irradiation techniques.³⁰⁻³¹ In this chapter, we explore a novel “grafting onto” method for synthesis of well-structured CS-*g*-PDMAEMA brush copolymer by combining ATRP with active ester conjugation techniques (the synthesis route was illustrated in Scheme 5.1), and its pH-responsive association behavior in aqueous solution was subsequently examined. Micellization behaviors of DMAEMA involving two hydrophilic block copolymers have been well studied, as reported by Armes et al for PDMAEMA-PDEAEMA copolymers³² and Gohy and coworkers for P2VP-PDMAEMA diblocks³³. In the later case, three different association states were observed depending on pH due to relatively large difference between the pK_a of the P2VP and PDMAEMA blocks. As an important representative of the pH-stimuli micelle component, PDMAEMA also has been assembled into many novel copolymers recently.³⁴⁻³⁸ By forming nanospheres or micelles via self-assembly, CS graft copolymers have attracted abroad interests because of their biomedical applications.³⁹⁻⁴⁰

Scheme 5.1 Synthesis route of CS graft copolymer via ATRP.

5.2 Experimental

5.2.1 Materials

CS oligomer ($M_n = 10$ kDa) with ca. 88.5 % degree of deacetylation (determined by ^1H NMR) was obtained from Haidebei Marline Bioengineering Co., Ltd., China. It was purified according to the description in Section 3.1.1.1. *N*-hydroxysuccinimide (NHS, 97%, Aldrich) was vacuum-dried at 60 °C for 24 h before usage. DMAEMA (98%, Aldrich) was purified by passing through a column of activated basic alumina and distilled under CaH_2 to remove inhibitors. Tetrahydrofuran (THF, 99.9%, Aldrich) and dichloromethane (DCM, 99.8%, Aldrich) were dried by sodium and anhydrous calcium chloride respectively and distilled. 2-Bromoisobutyryl bromide (2-BIB, 98 %, Aldrich), triethylamine (TEA, 99%, Aldrich), 1,1,4,7,10,10-hexamethyl triethylenetetramine (HMTETA, 97%, Aldrich), copper (I)

bromide (CuBr, 99.999%, Aldrich) and other reagents were used as received.

5.2.2 Synthesis of ATRP initiator

The initiator, *N*-(2-bromo-2-methylpropionyloxy) succinimide (NBPS), was prepared according to Lecolley et al's reports.⁴¹⁻⁴² Vacuum-dried NHS (3.45 g, 30 mmol) was dissolved in 50 mL anhydrous DCM with TEA (9.24 mL, 66 mmol) under argon in a 100 mL Schlenk flask. The flask was cooled to 0 °C with an ice bath, followed by dropwise addition of 2-BIB (4.09 mL, 33mmol, dissolved in 25 mL DCM). The mixture was stirred for 45 min and raised to room temperature for further 4 h reaction. The mixture was then poured into excess cold water. The organic layer was separated and washed with saturated sodium carbonate, HCl (pH 4.5), followed by saturated sodium carbonate aqueous solutions. The organic solution was dried over anhydrous sodium sulfate and filtered, and the DCM solvent was removed using a rotary evaporator under a reduced pressure. The product was crystallized from hexane and obtained as a light yellow solid (6.67 g, yield: 84 %).

5.2.3 Homopolymerization of DMAEMA via ATRP

The typical synthesis of linear PDMAEMA capped with *N*-succimidyl (NSI) group is described. A 25 mL Schlenk flask was connected to a vacuum line, and the moisture was removed by heating under vacuum. When the flask was cooled down, dry argon was filled back. Under an argon environment, the initiator NBPS (0.132 g, 0.5 mmol) and CuBr (0.072 g, 0.5 mmol) were charged into the flask that was tightly sealed with a rubber septum. Using the argon purged syringes, DMAEMA (8.425 mL,

50 mmol) and THF (8.7 mL) were introduced into the flask. The reaction mixture was degassed by three freeze-vacuum-thaw cycles. Thereafter, HMTETA (0.27 mL, 1.0 mmol) was injected into the system at room temperature and further degassed by three freeze-vacuum-thaw cycles. The reaction was allowed to proceed with constant stirring at 40 °C for 12 h. The polymerization was terminated and the solution was passed through a basic alumina column and eluted with additional 100 mL THF. After being condensed in a rotary evaporator, the solution was poured into excess amounts of cold hexane to precipitate the polymer. The product was isolated and dried under vacuum to yield the white powder (6.76 g, yield: 86 %). ¹H NMR (CDCl₃) δ(ppm): 4.08 (s, -O-CH₂CH₂N), 2.83 (s, -OC-CH₂CH₂-CO-), 2.60 (s, -O-CH₂CH₂N), 2.33 (s, -N(CH₃)₂), 2.31(s, -C(CH₃)₂-CO-), 1.85-1.97 (d, -C-CH₂-), 0.91-1.07 (d, -C-CH₃).

5.2.4 Synthesis of CS-g-PDMAEMA copolymer

The CS oligomer (300 mg, 0.03 mmol) was dissolved in 40 mL acetic acid buffer solution and degassed, and PDMAEMA (*M_n* ~ 5 kDa, 8 g, 1.6 mmol) was added gradually under argon atmosphere. Acetic acid was added to maintain the solution pH value to be around 6.5 and the mixture was stirred at 4 °C for 24 h. The product was dialyzed (MWCO = 8 kDa) against distilled water at 4 °C for 3 days and then lyophilized into a dry powder. The residual homopolymer PDMAEMA was removed by extraction with methanol for 24 h in a Soxhlet extractor, and the final product was isolated by filtration and dried under vacuum (572 mg, yield: 73 %).

5.2.5 Characterization

The ¹H NMR spectra were recorded using a Bruker DMX-400 spectrometer

with CDCl_3 , D_2O or $\text{D}_2\text{O}/\text{DCI}$ as solvent. Fourier transformed infrared (FTIR) transmission spectra were obtained on a Perkin-Elmer Spectrum 100 spectrometer by accumulation of 32 scans, with a resolution of 4 cm^{-1} . The samples for FTIR were prepared in KBr pellets.

Molecular weight and polydispersity of the polymers were determined by gel permeation chromatography (GPC) at $25\text{ }^\circ\text{C}$. For PDMAEMA, an Agilent 1100 series GPC system equipped with a LC pump, PLgel MIXED-C column and RI detector was used. The column was calibrated with Agilent EasiCal polystyrene standards of narrow molecular weight distribution. The HPLC grade THF containing 1 vol% TEA was used as a mobile phase and the flow rate was 1.0 mL/min .⁴³ For CS and its copolymer, the GPC column was changed to PL aquagel-OH Mixed, which was calibrated with Agilent EasiCal PEO standards. An acetic acid/sodium acetate buffer solution (0.2 M , $\text{pH } 2.7$) was used as eluent.⁴⁴

The potentiometric and conductimetric titrations were carried out on a Metrohm Titrando system equipped with a Conductometer 712. The water jacketed titration vessel was maintained at a constant temperature of $25\text{ }^\circ\text{C}$ using a circulating water bath. A 30 mL volume of $0.1\text{ wt\%}$ copolymer solution was prepared with an excess of 0.05 M HCl and titrated by 0.1 M NaOH under constant stirring.

Static light scattering (SLS) and dynamic light scattering (DLS) experiments were performed by means of a laser light scattering spectrometer (Brookhaven BI-200SM) equipped with an argon ion laser operating at $\lambda = 488\text{ nm}$ and a BI9000AT multi- τ digital time-correlator. For DLS, 90° measurement angle and 3 min

correlation measurement time were used as standard parameters. Distribution averages and particle size distributions were computed using cumulant analysis and CONTIN routes. All data were averaged over three measurements. The polymer solutions were prepared at the concentration of 0.05 wt% and filtered by 0.8 μm and 0.2 μm filters in tandem prior to the light scattering experiments.

The zeta potential of the polymer colloid system was characterized by a Brookhaven ZetaPALS analyzer. The measurements were performed at 25 °C under the Smoluchowski approximation, and 3 runs of 20 cycles were chosen for a good reproducibility.

The micrographs of the unimers and polymer aggregates were taken using a JEOL JEM-2010 transmission electron microscope (TEM) operating at an accelerating voltage of 200 kV. The TEM samples were prepared by dripping a polymer solution onto 400-mesh copper grids pre-coated with Formvar and stained by 0.2 wt% phosphotungstic acid prior to freeze-drying them.

The critical micelle concentration (cmc) of the CS-*g*-PDMAEMA copolymer was determined by measuring the surface tension using a Dataphysics DCAT 21 tensiometer equipped with a Wilhelmy plate at 25 °C. A 0.1 wt% graft copolymer solution was titrated into 50 mL acetic acid buffer with the same pH value and ionic strength as the titrant, and the surface tension was recorded and plotted against the polymer concentration.

5.3 Results and discussion

5.3.1 Synthesis of CS-*g*-PDMAEMA copolymer

As the first step to synthesize the CS-*g*-PDMAEMA, the NBPS initiator was prepared from the bromoacrylation of NHS. From the FTIR spectra (Figure 5.1), the disappearance of the broad absorption band between 2400~3500 cm⁻¹ indicates that the -OH group of NHS was substituted. The strong carbonyl absorption at 1740 cm⁻¹ indicates the formation of ester groups. The ¹H NMR spectrum of NBPS in Figure 5.2a yields the following chemical shifts: $\delta=2.09$ (s, 6H, -C(CH₃)₂-CO-), $\delta=2.88$ (s, 4H, -OC-CH₂-CH₂-CO-), which agree with the literature.⁴¹⁻⁴² The ratio of integral peak areas equals 1.5:1, which matches the expected number of protons, indicating the high purity of the initiator. The homopolymer PDMAEMA was synthesized by ATRP method and its typical ¹H NMR spectrum in CDCl₃ was also shown in Figure 5.2a(ii). From the spectrum, we can find all the characteristic signals of DMAEMA. The $M_{n,NMR}$ can be calculated from the integral ratio of “ I_b ” to “ I_i ”, according to

$$M_{n,NMR} = (2I_b / I_i) \times 157 + 264 \quad (5-1)$$

where I_b and I_i are the integral peak areas of $\delta(\text{ppm})=2.60$ (s, 2H, -O-CH₂CH₂N) from DMAEMA and $\delta(\text{ppm})=2.83$ (s, 4H, -OC-CH₂-CH₂-CO-) from NBPS initiator, respectively.

Figure 5.1 FTIR spectra of (a) NHS and (b) NBPS.

Subsequently, the PDMAEMA precursor was grafted onto the CS backbone via active ester conjugation method and the comb copolymer, CS-*g*-PDMAEMA, was obtained. Considering that the long DMAEMA segments may screen the NSI head group due to steric hindrance, thus reduce the conjugation efficiency, we chose PDMAEMA with M_n of 5 kDa for the subsequent synthesis of the graft copolymer. The polymerization time was limited within 8 h to prevent the functional end-group loss. To maximize the modification of amines and minimize the hydrolysis of NHS esters, the CS concentration was maintained at a relatively high concentration (> 5 mg/mL). The molar ratio of NSI capped PDMAEMA to amino groups of CS was kept near 1:1 to achieve a reasonable grafting efficiency, and the further increment of PDMAEMA molar ratio was curbed by its solubility. Due to the high pK_a (~ 7)⁴⁵ of DMAEMA, the graft copolymer CS-*g*-PDMAEMA was synthesized in a pH 6.5 acetic acid buffer solution to prevent the precipitation of CS. In order to guarantee the purity of the conjugated copolymer, the final product was re-extracted for another 24

h using Soxhlet extractor and vacuum-dried. No obvious weight reduction was observed.

Figure 5.2 (a) ^1H NMR spectra of: (i) NBPS and (ii) homo-PDMAEMA initiated by NBPS (CDCl_3) at 25 $^\circ\text{C}$. (b) ^1H NMR spectra of: (iii) CS (10k) (D_2O), (iv) CS-g-PDMAEMA ($\text{D}_2\text{O} + \text{DCl}$) at 25 $^\circ\text{C}$.

In the FTIR spectrum of the copolymer as shown in Figure 5.3a, the characteristic peaks appearing at 2820 cm^{-1} and 2768 cm^{-1} (C-H stretching of the $-\text{N}(\text{CH}_3)_2$), 1726 cm^{-1} (the carbonyl group), 1455 cm^{-1} (CH_2 bending), and 1150 cm^{-1}

(C-N stretching) suggest the presence of DMAEMA in the copolymer. The ^1H NMR spectra of the CS and CS-*g*-PDMAEMA were shown in Figure 5.2b. In the ^1H NMR spectrum of the copolymer, the characteristic chemical shifts of DMAEMA segments are readily identifiable: the peaks at 2.85, 3.44 and 4.25 ppm are attributed to the methyl protons, methylene protons on the tertiary amine, and the ester methylene protons, respectively. Compared with the ^1H NMR spectrum of PDMAEMA in CDCl_3 (Figure 5.2a), the former two peaks shifted from around 2.3 and 2.6 ppm to 2.8 and 3.4 ppm, respectively. This phenomenon can be explained by the electric field effect from the protonated neighboring tertiary amine when adding DCl to dissolve the copolymer.³² By comparing the intensities of the peaks at 3.4~3.7 ppm (m, glucosamine unit, H-3~6) with the methyl peak at 2.8 ppm, the graft ratio (= weight percentage of DMAEMA to the copolymer) can be calculated as 58 %.⁴⁴ The GPC curves also confirmed the growth of the molecular weight (Figure 5.3b): the copolymer $M_n = 26600$ g/mol and $M_w/M_n = 1.81$, which was close to the $M_{n,NMR} = 23700$ g/mol. Kang et al. reported that PDMAEMA grafted *N*-carboxyethyl CS achieved via ammonium persulfate initiated free radical polymerization can reach a graft ratio of about 50 %.³⁰ We can find the active ester conjugation technique provides a new grafting option for CS copolymer in mild conditions. Of course, compared with free radical polymerization and other active ester reaction taking place in organic solvent⁴⁶, the graft efficiency (= weight percentage of DMAEMA in copolymer to the charged PDMAEMA) was relatively low due to the hydrolysis effect in aqueous solution.

Figure 5.3 (a) FTIR spectra of: (i) CS, (ii) CS-g-PDMAEMA, and (iii) PDMAEMA. (b) GPC traces of CS and CS-g-PDMAEMA.

5.3.2 Kinetic studies on the ATRP process

The ATRP results of DMAEMA initiated by NBPS were summarized in Table 5.1. In order to obtain distinct molecular weight growth during the synthesis process, the polymers (e.g. SM3 and SM4) with the larger molar ratios of monomer to initiator were chosen for the kinetic studies by periodically extracting aliquot solutions from

the reaction vessel using long needle syringes. The GPC curves of SM3 at various time intervals of reaction were presented in Figure 5.4a, showing the smooth increment of molar mass with a narrow distribution. The corresponding M_n and M_w / M_n versus % conversion were plotted in Figure 5.4b. The linear relationship between $M_{n,GPC}$ and % conversion indicates that the reaction occurred under controlled/“living” conditions. This is further supported by the linear first-order kinetic plot shown in Figure 5.5a. At a higher temperature of 60 °C under the identical conditions (SM4), the polymerization remained well-controlled. The apparent first-order rate constant, k_{app} , increased from 0.364 h⁻¹ at 40 °C to 0.892 h⁻¹ at 60 °C (Figure 5.5b), which indicates the higher polymerization rate at the higher temperature due to the increase in both the radical propagation rate and the atom transfer equilibrium constant. The lower monomer conversion and the slightly bigger M_w / M_n achieved at 60 °C may be attributed to the increased chain transfer at the elevated temperature.

Table 5.1 Synthesis of PDMAEMA using NBPS as initiator via ATRP^a

No.	[I] ₀ : [M] ₀	Temp. (°C)	Time (h)	Conv. (%)	$M_{n,theo}$ ^b (g/mol)	$M_{n,NMR}$ ^c (g/mol)	$M_{n,GPC}$ (g/mol)	M_w / M_n ^d
SM1	1:15	40	8	94	2 480	2 500	2 300	1.16
SM2	1:30	40	8	92	4 600	5 200	5500	1.14
SM3	1:100	40	12	86	13 800	14 000	15 700	1.11
SM4	1:100	60	8	83	13 300	13 800	14 800	1.21

^a Other conditions: [CuBr]₀: [HMTETA]₀: [NBPS]₀=1:2:1, [NBPS]₀=0.5 mmol, 50 vol% THF.

^b Calculated according to $M_{n,theo} = \left\{ (Conv. \times [DMAEMA]_0 \times 157) / [Initiator]_0 \right\} + 264$, where 157 and 264 are the molecular weights of DMAEMA monomer and NBPS initiator, respectively.

^c Determined by eq.(5-1).

^d Measured by GPC, PSt standards.

Figure 5.4 (a) GPC traces for homopolymerization of DMAEMA (SM 3) in 50 vol% THF at various reaction times. (b) Evolution of experimental M_n (dash line is theoretical value) and PDI with conversion. $[\text{CuBr}]_0$: $[\text{HMTETA}]_0$: $[\text{NBPS}]_0 = 1:2:1$, $[\text{NBPS}]_0 = 0.5$ mmol, $[\text{DMAEMA}]_0 = 50$ mmol, and $T = 40$ °C.

Figure 5.5 (a) Semilogarithmic kinetic and conversion plot for ATRP of DMAEMA at $T = 40\text{ }^{\circ}\text{C}$.
 (b) Semilogarithmic kinetic plots of ATRP of DMAEMA at $T = 40, 60\text{ }^{\circ}\text{C}$ respectively.
 $[\text{CuBr}]_0:[\text{HMTETA}]_0:[\text{NBPS}]_0 = 1:2:1$, $[\text{NBPS}]_0 = 0.5\text{ mmol}$, $[\text{DMAEMA}]_0 = 50\text{ mmol}$ in 50 vol% THF.

5.3.3 Association behavior of CS-g-PDMAEMA

Since the graft copolymer carries primary and tertiary amine groups on the CS and DMAEMA segments respectively, it shows significant pH-sensitive properties. We conducted the titration experiments by adding 0.1 M NaOH into 0.1 wt% CS-g-PDMAEMA dissolved in a 0.05 M HCl solution. From the pH and conductivity titration curve (Figure 5.6a), three regions can be distinguished and two inflection points (point A at pH ~ 4.0; point B at pH ~ 8.5) were observed. At the beginning at pH < 2, the polymer solution contained H⁺, Na⁺, OH⁻, Cl⁻, and fully protonated CS and DMAEMA segments. With the addition of NaOH, the concentration of H⁺ decreased while the Na⁺ increased. Since the mobility of Na⁺ is much smaller than that of H⁺, the conductivity of the polymer solution decreased to the first inflection point A while the pH value increased.⁴⁷ The primary amine groups on the CS firstly deprotonated at point A and followed by the tertiary amine groups on the DMAEMA segments with the continuous base addition. The increase of Na⁺ concentration led to the progressive increase in the conductivity until point B. The second inflection point B indicates the end of deprotonation of tertiary amine groups on the DMAEMA segments, and pH and conductivity increased with further addition of base after that. In order to examine the proton dissociation of the weak basic polyelectrolytes and their corresponding association behaviors when changing pH, the titrations into pure CS and PDMAEMA solutions were also carried out and their potentiometric data were plotted against the fraction of charged amine (α) in Figure 5.6b. Here, α ranged from 0 at the unionized state of amine groups to 1 at the fully protonated state. At the

same pH, we found that the α values of PDMAEMA were larger than that of CS. For example, at pH 5 and 6, α values of PDMAEMA are 0.92 and 0.64, whereas CS's are 0.68 and 0.32 correspondingly.

Figure 5.6 (a) Potentiometric and conductimetric titration curves of 0.1 wt% CS-g-PDMAEMA copolymer in an excess of 0.05 M HCl solution. The inset represents the difference plot of the pH curve. (b) Comparison of pH values versus protonation degree of pure CS and PDMAEMA.

Figure 5.7 (a) Hydrodynamic radius, R_h , and the ratio of R_g / R_h as a function of pH of CS-g-PDMAEMA copolymer in aqueous solution. Concentration of copolymer was fixed at 0.5 mg/mL in all the solutions. (b) Zeta potential results of CS-g-PDMAEMA copolymer at different pH values.

The pH-responsive association behavior of CS-g-PDMAEMA in aqueous

solution was investigated where the intensity-average hydrodynamic radius (R_h) and gyration radius (R_g) of the copolymer were measured by DLS and SLS, respectively. As depicted in Figure 5.7a, the R_h values increased from about 47 nm to 252 nm (with scattered light intensity increased from 30 kcps to 1.9 mcps) and the R_g / R_h values decreased from 1.42 to 0.56, when the pH was changed from 4 to 8. As we know, the dimensionless ratio R_g / R_h , is a characteristic parameter describing the conformation of a polymer chain in solution and can be further applied to elucidate the micellar structure. The theoretical values of R_g / R_h for hard sphere micelle, core-shell structure, and random coils were reported to be 0.77, 0.90 and 1.5 respectively.⁴⁸⁻⁴⁹ Therefore, it can be concluded that the copolymer conformation changed from independent unimers to aggregates when the pH value varied, which was also reflected from the sharp scattered light intensity increment. The polydispersities of the aggregates, as evaluated by the ratio μ_2 / Γ^2 from cumulants analysis, were relatively narrow (< 0.1), which should ascribe to the narrow dispersity of the graft chains synthesized via ATRP.

Based on the potentiometric titrations of CS and PDMAEMA (Figure 5.6b) and light scattering results, four pH zones were divided, which corresponded to different conformations of the copolymer described as follows. (i) At pH ~ 4 , both CS and DMAEMA segments were almost fully protonated and the copolymer chains extended due to the strong electrostatic repulsion, so that the R_g / R_h value of 1.42 was very close to that of a Gaussian chain. (ii) At pH 5~6, a majority of CS segments were deprotonated and their hydrophobicity increased. At the same time, the

decreased electrostatic repulsion also facilitated hydrogen-bonded complex among CS domain,⁵⁰ resulting in the formation of micelles with CS in the core and the partially deprotonated DMAEMA segments as the corona. The R_g/R_h values (0.80~0.87) indicates that the core-shell structure was achieved and the slight increase in R_h upon the pH increment could be explained by the growth of the hydrophobic core size when the degree of deprotonation increased. (iii) At pH ~ 7, a majority of DMAEMA and almost all of the CS segments were deprotonated, and the double-layered hard spheres were formed with $R_g/R_h = 0.65$, which is in good agreement with the recently reported DMAEMA copolymer micelles³⁸. (iv) At pH ~ 8, almost fully deprotonated copolymer micelles aggregated and precipitated gradually due to the lack of sufficient electrostatic repulsion. Figure 5.7b shows the zeta potential change at different pH values. At pH 4, zeta potential was more than +30 mV, and it decreased step-by-step to near +1 mV when pH value was increased to 8, which indicates the ongoing deprotonation process of the copolymer.

The TEM observation further provided the straightforward evidence to support the conformation changes. Figure 5.8a shows the TEM micrograph of the copolymer at pH 4, where the copolymer unimers randomly dispersed as coil-like structure and no aggregation occurred. The TEM micrograph of the sample at pH 5 is shown in Figure 5.8b where spherical micelles were observed with a diameter of about 120 nm. The uneven darkness in the micelles suggests that these structures are core-shell micelles. When pH value reached 7, as shown in Figure 5.8c, the density of the micelles appears more uniform and higher, indicating that the hard spheres were

formed. The ratios between standard deviation and mean particle size calculated from the TEM statistic software were smaller than 0.2, which was in good agreement with the polydispersities obtained from the DLS experiments. Again, the importance of introducing ATRP to prepare CS copolymer was confirmed: well-defined molecular structures give uniform macroaggregates. On the basis of the experimental evidences from TEM and LLS, the conformational changes of CS-g-PDMAEMA at different pH values were depicted in Scheme 5.2.

Figure 5.8 TEM micrographs of 0.05 wt% CS-g-PDMAEMA copolymer at: (a) pH 4, (b) pH 5, and (c) pH 7.

Scheme 5.2 Schematic representation of the microstructure of CS-*g*-PDMAEMA copolymer at different pH values.

For a self-assembling system, the cmc is an important physical parameter usually used to describe the micellization behavior. We measured the cmc of the CS-*g*-PDMAEMA copolymer at different pH values from its surface tension variations with polymer concentration. The cmc values of the copolymer were 128, 117, and 79 mg/L at pH 5, 6 and 7, respectively. It is noteworthy that these values are significantly larger than the cmc values of another reported CS graft copolymer, polylactide (PLA)-*g*-CS, where PLA acted as the hydrophobic segments.⁴⁰ This difference shows that the hydrophilic/hydrophobic contrast between PDMAEMA and CS is less remarkable than that of the PLA/CS pair.

5.4 Summary

A well-defined comb-like graft copolymer, CS-*g*-PDMAEMA, was successfully synthesized under mild conditions by means of atom transfer radical polymerization (ATRP) and active ester conjugation. The successful application of ATRP endowed a narrow molecular weight distribution to the PDMAEMA side chains

of the copolymer, and the products were characterized by FTIR, ^1H NMR and GPC. The kinetic studies on the polymerization of DMAEMA initiated by NBPS revealed that the reaction was well controlled and that well-defined PDMAEMA was obtained. The graft ratio of DMAEMA to copolymer was 58 %, which is comparable to those of free radical polymerized CS graft copolymers. The association behavior of the graft copolymer in aqueous solution varied significantly upon changing the pH of the media, as investigated by LLS and TEM. At pH $\sim$ 4, the copolymers behaved as Gaussian chains; at pH 5~6, the core-shell structured micelles were achieved via self-assembly; at pH $\sim$ 7, the core-shell structure further collapsed into double-layered hard spheres; and at pH $\sim$ 8, the micelles aggregated and precipitated. The low polydispersity of the aggregates benefits from the narrow unimodal distribution of the side chains. The potentiometric titrations and zeta potential tests were also carried out to understand and verify the factors leading to these interesting phenomena.

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Chapter 6

Dual Hydrophilic Graft Chitosan Terpolymer Synthesized via ATRP and Click Chemistry

6.1 Introduction

The use of a “living” polymerization technique has led to better control of the formation of block or graft copolymers with well-defined structures, thus benefiting the understanding of structure-property relationships. Graft copolymerization of vinyl monomers onto chitosan (CS) and other polysaccharides using atom transfer radical polymerization (ATRP) have already been introduced. For example, methoxy capped (PEG 350) methacrylate was polymerized from a CS macroinitiator that was prepared by modifying CS with 2-bromoisobutryl bromide (2-BIB).¹ Polyacrylamide and polystyrene were successfully grafted to CS particles using heterogeneously surface-initiated ATRP. Other kinds of polysaccharides, such as cellulose, pullulan, dextran and β -cyclodextrin (β -CD) have also been modified via ATRP.²⁻⁵ Sui et al. recently reported that the homogeneous graft polymerization via ATRP from cellulose was accomplished by preparing a macroinitiator in an ionic liquid,⁶ but an ionic liquid suitable for modification of CS via ATRP still remains unavailable. A “grafting onto” approach seems more promising under such a background and several water-soluble graft CS copolymers have been synthesized using different coupling techniques.⁷⁻⁸ In particular, the active ester conjugation method is used effectively and has triggered more and more applications to synthesize graft CS copolymers. When combining the

active ester conjugation with ATRP, one precursor can lead to many functional polymers of different chemical functionality. Under these circumstances, we have successfully synthesized a pH-sensitive PDMAEMA grafted CS copolymer and evaluated its special association behavior in Chapter 5.⁹ Interestingly, Munro et al. also reported the synthesis of CS-g-poly(oligoethylene glycol methacrylate) (OEGMA) copolymers using ATRP and active esters, and concluded that the “grafting onto” synthetic route was preferable to the “grafting from” route due to the higher purity and more defined structure of the resulting polymer.¹⁰

In the past few years, “click chemistry”, as termed by Sharpless et al.,¹¹⁻¹³ has been proven to be a versatile method in polymer chemistry due to its high specificity, nearly quantitative yield, compatibility to a variety of functional groups, solvent insensitivity (also highly active in water), and applicability under mild conditions. More recently, the combination of click reactions with ATRP has aroused enormous interest and generated hundreds of research papers.¹⁴⁻¹⁷ In principle, three main strategies, which were summarized by Binder and Sachsenhofer, can be applied: the initiator approach, the Br^-/N_3^- approach and side-chain modifications.¹⁷ Especially for the last route, well-defined brush copolymers with a high grafting efficiency have been synthesized by coupling ATRP “grafting from” and click “grafting onto” techniques.¹⁸⁻¹⁹ There are already a few publications utilizing click chemistry in CS modifications, but further combination with ATRP has not been conducted. Kulbokaite et al. synthesized CS-methoxy poly(ethylene glycol) (MPEG) derivatives containing triazolyl moiety via 1,3-dipolar cycloaddition between pendant azide and

end alkyne groups of CS and MPEG, respectively.²⁰ By introducing a cationic group on CS, Gao et al. obtained water-soluble 6-*N,N,N*-trimethyltriazole-CS (TCS) that showed strong DNA binding ability and high protection of DNA against nuclease degradation.²¹ In a more detailed manner, Lallana et al. achieved the functionalizations of CS-*g*-PEG-*N*₃ via Cu(I)-catalyzed and strain-promoted azide-alkyne [3+2] cycloadditions (CuAAC and SPAAC), respectively.²²

Dual hydrophilic copolymers, which consist of two different hydrophilic blocks, can self-assemble into one or more types of micelles in aqueous media, when the hydrophilicity of one block changes differently from the other blocks, corresponding to a change in temperature, pH, or ionic strength, and this kind of association behavior was often metaphorized into “schizophrenic” micellization.²³⁻²⁵ It has also been found that the architecture of a dual hydrophilic copolymer can play an important role in determining its association behavior.²⁶⁻²⁷ Some novel dual hydrophilic copolymers with different architectures have been developed and their association properties were investigated.²⁸⁻³² Liu and coworkers have made significant contributions in this area. Specifically, they synthesized the linear and miktoarm star diblock copolymers poly(*N*-isopropylacrylamide)-*b*-poly[(2-diethylamino)ethyl methacrylate] (PNIPAM-*b*-PDEAEMA and PNIPAM-*b*-PDEAEMA₄),²⁸ the miktoarm star terpolymer PEO(-*b*-PDEAEMA)-*b*-PNIPAM,²⁹ and the Janus-type heteroarm star copolymer PDEAEMA-*CD*-PNIPAM.³⁰ They also fabricated the temperature- and pH-responsive four-layered nanoparticles from a POEGMA-*b*-PDMAEMA-*b*-PDEAEMA triblock copolymer and PNIPAM.³¹ Feng et al. recently synthesized a

well-defined graft copolymer, poly(*N*-isopropylacrylamide)-*b*-[poly(ethyl acrylate)-*g*-poly(2-vinylpyridine)] [PNIPAM-*b*-(PEA-*g*-P2VP)], and studied its micellization behavior using ¹H NMR, fluorescence spectroscopy, dynamic light scattering and transmission electron microscopy.³²

In this chapter, we report the synthesis of a comb-like graft CS copolymer via ATRP and click chemistry, and its association behavior in aqueous solutions. To the best of our knowledge, this work represents the first time of synthesis of a dual hydrophilic graft CS terpolymer and study of its thermo- and pH-responsive association behaviors. By self-assembling into nanospheres or micelles, dual hydrophilic graft CS copolymers are finding extensive applications in the field of biomedical science and engineering.³³⁻³⁴ This unique graft CS copolymer is believed to have a great potential in application for gene/drug delivery and controlled release.

6.2 Experimental

6.2.1 Materials

CS (M_n = 10 kDa) with ~89 % degree of deacetylation (DD, determined by ¹H NMR) was obtained from Haidebei Marline Bioengineering Co., Ltd., and it was purified according to the method reported in previous chapters. *N*-isopropylacrylamide (NIPAM, 97%, Aldrich) was recrystallized from a mixture of *n*-hexane and benzene (3:1, v/v). 2-(Dimethylamino)ethyl methacrylate (DMAEMA, 98%, Aldrich) was purified by passing through a column filled with activated basic alumina and distilled under calcium hydride (CaH₂) to remove inhibitors. Tetrahydrofuran (THF), isopropanol (IPA) and *N, N'*-dimethylformamide (DMF) were

dried and distilled prior to use. Ethyl 2-chloropropionate (ECP, 97%), Ethyl 2-bromoisobutyrate (EBIB, 98%), 1,1,4,7,10,10-hexamethyl triethylenetetramine (HMTETA, 97%), copper (I) bromide (CuBr, 99.999%), copper (I) chloride (CuCl, 99.995%), sodium azide (NaN₃, 99%), 2-(*N*-morpholino)ethanesulfonic acid (MES, 99%), sodium L-ascorbate (98%), copper (II) sulfate pentahydrate (CuSO₄·5H₂O, 98%), 4-pentynoic acid (95%), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl, 99%), *N*-hydroxysuccinimide (NHS, 97%) were purchased from Aldrich and used as received. Tris(2-(dimethylamino)ethyl)amine (Me₆TREN, 98%) was obtained from ATRP Solutions Inc. and used directly.

6.2.2 Synthesis of azido-terminated PDMAEMA homopolymer (PDMAEMA-*N*₃)

The typical synthesis of linear PDMAEMA capped with an azide group is described as follows. A 25 mL Schlenk flask equipped with a magnetic stirrer was connected to a vacuum line, and the moisture was removed by heating under vacuum. When the flask was cooled down, dry argon was filled in. Under an argon atmosphere, DMAEMA (2.52 mL, 15 mmol), THF (2.5 mL) and CuBr (0.072 g, 0.5 mmol) were charged into the flask that was tightly sealed with a rubber septum. The reaction mixture was degassed by three freeze-vacuum-thaw cycles. Thereafter, HMTETA (0.27 mL, 1.0 mmol) was injected into the system at room temperature and further degassed by three freeze-vacuum-thaw cycles. Using an argon purged syringe, the initiator EBIB (0.074 mL, 0.5 mmol) was introduced into the flask to start the ATRP. The reaction was allowed to proceed with constant stirring at 40 °C for 6 h. The polymerization was then terminated and the solution was passed through a basic

alumina column and eluted with additional 100 mL THF. After being condensed in a rotary evaporator, the solution was poured into excess amounts of cold hexane to precipitate the polymer. The above dissolution-precipitation cycle was repeated twice to remove residual monomer. The final viscous product was isolated and dried under vacuum. The yield was equivalent to 76% conversion of the monomer (1.79 g), $M_{n, GPC} = 4600$ g/mol and $M_w/M_n=1.22$. The actual degree of polymerization (DP) of PDMAEMA was obtained from the relative integration of characteristic protons of chain ends to protons of main chains by ^1H NMR analysis. Thus, the homopolymer obtained was denoted as PDMAEMA₂₃-Br.

The resulting homopolymer PDMAEMA₂₃-Br (1.72 g, 0.45 mmol) and NaN₃ (0.058 g, 0.9 mmol) were dissolved into 25 mL DMF in a 50 mL round-bottomed flask. The mixture was allowed to react under stirring at room temperature for 24 h. After removing the DMF solvent under vacuum, the residue was redissolved in 50 mL THF and passed through a basic alumina column to remove sodium salt and excess NaN₃. The filtrate, after condensed to a volume of ~2 mL by rotary evaporation, was precipitated in 50 mL cold hexane. The precipitate was filtered, washed by hexane and vacuum-dried. The yield was 96% (1.65 g), $M_{n, GPC}=4600$ g/mol and $M_w/M_n=1.22$.

6.2.3 Synthesis of azido-terminated PNIPAM homopolymer (PNIPAM-*N*₃)

The experiment procedure of ATRP for the synthesis of PNIPAM-*N*₃ was similar to the preparation of PDMAEMA. A typical mixture containing CuCl (0.05 g, 0.5 mmol), NIPAM (2.82 g, 25 mmol), degassed IPA (7.2 mL), Me₆TREN (0.116 g, 0.5 mmol) and ECP (0.064 mL, 0.5 mmol) reacted at room temperature for 10 h. The

flask was quenched into liquid nitrogen to terminate the polymerization and exposed to air. After evaporating most of IPA under reduced pressure, the residue was diluted with THF and passed through a neutral alumina column to remove the copper catalyst. Then, the solution was concentrated into around 3 mL and PNIPAM-Cl was precipitated with an excess of diethyl ether. The obtained solid was filtered, washed and dried under vacuum to give a yield of 78% (2.2 g). The GPC analysis revealed $M_n = 5600$ g/mol and $M_w/M_n = 1.12$. The actual DP of the PNIPAM block was determined to be 40 by ^1H NMR analysis in D_2O (comparing the peak area for NIPAM isopropyl at 3.9 ppm with that for the ethoxy at 4.1 ppm). The homopolymer obtained was denoted as PNIPAM₄₀-Cl.

In order to prepare the azido-terminated PNIPAM homopolymer, the resulting PNIPAM₄₀-Cl (2.12 g, 0.47 mmol) and NaN_3 (0.062 g, 0.94 mmol) were dissolved into 10 mL DMF in a 25 mL round-bottomed flask. The mixture was allowed to react under stirring at 45 °C for 48 h. After removing DMF under vacuum, the residue was redissolved in 50 mL THF and passed through a neutral alumina column to remove sodium salt and excess NaN_3 . The filtrate was concentrated by rotary evaporation, and precipitated in 50 mL diethyl ether. The precipitate was filtered, washed by diethyl ether and vacuum-dried. The yield was 86% (1.83 g), $M_{n,\text{GPC}} = 5600$ g/mol and $M_w/M_n = 1.12$.

6.2.4 Synthesis of alkynyl-pendant CS (*alkynyl*-CS)

The precursor for the click reaction, *alkynyl*-CS, was prepared by the amidation of CS with 4-pentynoic acid in the presence of EDC/NHS. In a typical

procedure, CS (0.5 g, 2.73 mmol) and 4-pentynoic acid (0.268 g, 2.73 mmol) were dissolved in 50 mL of MES buffer (0.1 M, pH adjusted to 5) and degassed. Being protected by argon, EDC (1.57 g, 8.19 mmol) and NHS (2.825 g, 24.57 mmol) were gradually charged into the flask within 20 min. The reaction was conducted at room temperature under stirring for 16 h. To remove impurities, the polymer solution was dialyzed (MWCO = 3 kDa) against distilled water for 3 days at 4 °C and freeze-dried. The yield was 87% (0.55 g), $M_{n, GPC} = 13800$ g/mol and $M_w/M_n = 1.64$.

6.2.5 Synthesis of CS(-g-PDMAEMA)-g-PNIPAM terpolymer

To a 50 mL Schlenk flask, *alkynyl*-CS (0.25 g, 0.6 mmol), PDMAEMA- N_3 (1.52 g, 0.4 mmol) and PNIPAM- N_3 (1.8 g, 0.4 mmol) was dissolved in 20 mL 0.1 M HCl solution and degassed. Under an argon atmosphere, $CuSO_4 \cdot 5H_2O$ (0.3 g, 1.2 mmol, in 3 mL of water) and sodium L-ascorbate (1.19 g, 6 mmol, in 5 mL of water) were successively added. Then, the pH value of the solution was adjusted to about 6.5 (aq NaOH). The yellow-colored mixture was stirred for 24 h at room temperature. The product was dialyzed (MWCO = 8 kDa) against 0.1 % acetic acid for 1 day and against distilled water for 3 days at 4 °C, and then lyophilized into dry powder. The residual homopolymers PDMAEMA and PNIPAM were removed by extraction with isobutyl alcohol for 24 h in a Soxhlet extractor, and the final product was isolated by filtration and dried under vacuum (1.92 g, overall yield: 77%).

6.2.6 Characterization

The procedure used to characterize the samples by 1H NMR, FTIR,

potentiometric titrations, SLS and DLS, zeta potentials, TEM, and critical micelle concentration (cmc) experiments has been described in Section 5.2.5.

Molecular weight and polydispersity of the polymers were also determined using GPC at 25 °C. For PDMAEMA and PNIPAM homopolymers, an Agilent 1100 series GPC system equipped with a LC pump, PLgel columns and RI detector was used. The columns were calibrated with polystyrene standards (Agilent EasiCal). The HPLC grade THF containing 1 % (v/v) triethylamine (TEA) and 0.25 % (w/v) tetrabutylammonium bromide (TBABr) were used as a mobile phase for PDMAEMA and PNIPAM, respectively. For CS and its derivatives, the GPC column was replaced by PL aquagel-OH Mixed, which was calibrated with pullulan standards. An acetic acid/sodium acetate buffer solution (0.2 M, pH 2.7) was used as eluent. The flow rate was fixed at 1.0 mL/min.

Scheme 6.1 Synthetic route for preparation of CS(-g-PDMAEMA)-g-PNIPAM graft terpolymer.

6.3 Results and discussion

6.3.1 Synthesis of CS(-g-PDMAEMA)-g-PNIPAM terpolymer

As shown in Scheme 6.1, a three-step approach was employed in the terpolymer synthesis. It proceeded first with the synthesis of homopolymers of

DMAEMA and NIPAM via ATRP, followed by substituting the halide end groups with azido groups, and subsequently grafting PDMAEMA- N_3 and PNIPAM- N_3 side chains onto the alkyne-containing CS backbone via click chemistry, resulting in the target terpolymer, CS(-*g*-PDMAEMA)-*g*-PNIPAM.

ATRP has been proved to be a versatile technique for the controlled polymerization of many hydrophilic monomers, and the ATRP of DMAEMA in protic and aprotic solvents have been well documented.^{9,35-36} The synthesis of PNIPAM via ATRP basically followed the similar procedures initiated by Stöver et al.³⁷ The subsequent azidation of homopolymers with NaN₃ leading to PDMAEMA- N_3 and PNIPAM- N_3 was carried out by referring to the previous reports.^{31,38} The FTIR spectra of PDMAEMA and PNIPAM after azidation (data not shown) clearly reveal the new appearance of an absorbance peak around 2100 cm⁻¹, which is characteristic of a terminal azido group.^{29,39} Nevertheless, the azido group substitution did not affect the molecular weights and distributions of homopolymers. It should be noted that, the different small molecules indicated in the experimental section were added in the THF mobile phase for GPC characterizations in order to minimize the column absorption and polymer aggregation phenomena.

Being utilized as a carboxyl activating reagent, EDC can initiate the formation of an amide linkage between 4-pentynoic acid and CS by forming an active intermediate, and NHS can increase the stability of the active intermediate.^{7,40} In the FTIR spectrum of *alkynyl*-CS precursor shown in Figure 6.1, the characteristic absorbance peak at ~2120 cm⁻¹ suggests the successful introduction of alkynyl group

on CS backbone.⁴¹⁻⁴² As compared with the CS spectrum, other newly appeared or strengthened absorbance peaks around 3250, 1400 and 630 cm⁻¹ represent the alkyne C-H stretching, alkane C-H bending and alkyne C-H bending, respectively. The ¹H NMR spectra of CS and *alkynyl*-CS (2% DCl in D₂O) are shown in Figure 6.2. The new signals appearing at δ =2.2–2.5 ppm can be ascribed to methylene protons (4H, -CH₂-CH₂-), and the alkynyl proton (1H, -C $\equiv$ CH) signal at δ =1.9 ppm is overlapped with that of CS acetyl groups.^{18,43} The degree of substitution (DS = 0.5) of *alkynyl*-CS was determined by the peak integral ratio of resonance signals in the range of 2.2–2.5 ppm to that at 2.9 ppm (H₂ of CS). Serving as an alternative method, potentiometric titration also can yield the DS by gradually neutralizing protonated amino groups along the *alkynyl*-CS backbone. The experiments were conducted by titrating 30 mL 0.1 wt% polymer solutions (dissolved in 0.05 M HCl) with 0.1 M NaOH. As shown in Figure 6.3, the consumption of titrant NaOH between two inflection points for *alkynyl*-CS is smaller than that of CS, which means the *alkynyl*-CS has a lower content of amino group due to the amidation. The experiments were conducted by titrating 30 mL 0.1 wt% polymer solutions (dissolved in 0.05 M HCl) with 0.1 M NaOH, and the molar amount of amino groups within the copolymer is equal to that of titrated base. The calculation was based on Equation 6.1.

$$\frac{30 \times (0.89 - x)}{[244x + 162 \times (0.89 - x) + 202 \times 0.11]} = 0.07 \quad (6-1)$$

where x is the DS, and 162, 202 and 244 corresponds to the unit molecular weight for glucosamine, acetyl-glucosamine and alkynyl-glucosamine, respectively. Equation 6.1

yields $x = 0.42$, which is very close to the DS obtained from ^1H NMR. The aqueous GPC traces for CS and *alkynyl*-CS are displayed in Figure 6.6, revealing a similar peak shape with M_n of 10200 (M_w/M_n of 1.71) and 13800 g/mol (M_w/M_n of 1.64) correspondingly. The GPC results not only reconfirm the successful incorporation of alkynyl groups, but also agree with the NMR result, $M_{n,\text{NMR}} = 12600$ g/mol, for *alkynyl*-CS.

Figure 6.1 FTIR spectra of CS and *alkynyl*-CS. The inset represents the magnified spectrograms in the specific wavenumber range.

Figure 6.2 ^1H NMR spectra recorded in $\text{D}_2\text{O}/\text{DCI}$ for CS and *alkynyl*-CS.

Figure 6.3 Potentiometric titration curves for 0.1 wt% CS and *alkynyl*-CS in an excess of 0.05 M HCl solution.

In the final step, the synthesis of the CS graft terpolymer was accomplished by the click reaction of *alkynyl*-CS with PDMAEMA- N_3 and PNIPAM- N_3 under mild conditions. In order to increase the grafting efficiency and obtain a balanced dual-

responsive copolymer during the “grafting onto” process, the lengths of the linear side chains were deliberately manipulated to reduce the steric congestion.¹⁸ Meanwhile, the pH value was adjusted to ~6.5 to minimize the electrostatic repulsion between the PDMAEMA and CS blocks, and the solubility of CS can also be maintained in this range because the incorporation of alkynyl groups destroyed the strong inter- and intra-macromolecular hydrogen bonds. An excess of azido-terminated homopolymers was used to ensure the complete consumption of alkynyl moieties, and the removal of excess homopolymers was achieved by dialysis and extraction. The FTIR spectrum of the purified product clearly reveals the presence of absorbance peaks of all three components (Figure 6.4c): 3420 cm^{-1} (O-H stretching) from CS; 1725 cm^{-1} (ester stretching), 1460 cm^{-1} (CH_2 bending), and 1145 cm^{-1} (C-N stretching) from DMAEMA; and 2970 cm^{-1} (C-H stretching), 1645 cm^{-1} (carbonyl stretching), and 1545 cm^{-1} (N-H bending) from NIPAM. Comparing the FTIR spectra among *alkynyl-CS*, *PDMAEMA-N₃*, and *PNIPAM-N₃*, the most noteworthy change is the complete disappearance of the absorbance peaks characteristic of the azido and alkynyl groups at ~2100 cm^{-1} . Based on this observation, two important conclusions can be drawn: the alkynyl moieties are fully attached to the homopolymeric side chains, and excess azido-terminated homopolymers have been thoroughly removed.^{29,39,44}

From the ^1H NMR spectrum of the CS graft terpolymer (Figure 6.5e), all characteristic signals of PDMAEMA, PNIPAM and CS segments can be discerned, which are accompanied by the appearance of a new resonance peak j at ~8.1 ppm (methine proton of 1,2,3-triazole ring).⁴⁵ The average constitution of the terpolymer

was determined by calculating the integral ratios between non-overlapping peaks belonging to each block: H2 at $\delta = 3.1$ ppm from CS, methylene protons (2H, -CH₂-CH₂-N-) at $\delta = 3.45$ ppm from PDMAEMA and tertiary proton (1H, -NH-CH-) at $\delta = 3.75$ ppm from PNIPAM, respectively. Assuming that no degradation occurred during the click reaction, the $M_{n,NMR}$ of the terpolymer can reach 130000 g/mol. The GPC eluograms (Figure 6.6) indeed show that the graft terpolymer has larger M_n (= 54000 g/mol) and smaller M_w/M_n (= 1.33) than these of CS and *alkynyl*-CS, which is inconsistent with the recent reports showing serious CS backbone breakdown under “clicking” in the presence of Cu(II)/ascorbate.^{20,22}

It has been proposed that the depolymerization of CS was catalyzed by the hydroxyl radicals ($\cdot$ OH), and the amino groups of CS may facilitate the depolymerization by favoring the generation of $\cdot$ OH close to the Cu-NH₂ binding site.²² This explains why CS with a lower content of amino group was more stable against degradation.²⁰ Therefore, CS(-g-PDMAEMA)-g-PNIPAM terpolymer synthesized from *alkynyl*-CS with a real DD of 39% revealed inconspicuous degradation compared to other studies^{20,22} and reconfirmed the above proposed mechanism. From another point of view, the disparity between the theoretical and GPC measured number average molecular weights of terpolymer may be attributed to the more compact nature of comb-like copolymers in good solvents.⁴⁶⁻⁴⁷ Alfred et al. also reported that the GPC underestimated the molecular weights of polymerized norbornene or oxanorbornene macromonomers grafted with PEO recently.⁴⁸ The low M_w/M_n of the terpolymer, indicating a high structural regularity, mainly benefited

from the inclusion of narrow-distributed side chains: PDMAEMA and PNIPAM. Of course, the dialysis process that eliminated the polymer chains with the lowest range of molecular weights also helped to improve the uniformity.

On the basis of the above results, we can conclude that the obtained graft CS terpolymer has a statistically well-defined structure: PDMAEMA side chains (23 repeating units) and PNIPAM side chains (40 repeating units) were attached to 28% and 22% of the glucosamine units of CS backbone, respectively, and the CS backbone also has 39% glucosamine and 11% acetyl-glucosamine units. In the subsequent section, we further investigate its pH- and thermo-responsive micellization behavior in different aqueous solutions.

Figure 6.4 FTIR spectra of (a) PDMAEMA- N_3 , (b) PNIPAM- N_3 , and (c) CS(-g-PDMAEMA)-g-PNIPAM.

Figure 6.5 ^1H NMR partial spectra of CS(-g-PDMAEMA)-g-PNIPAM recorded in D_2O under different conditions: (a) pH 4 and 25 $^\circ\text{C}$, (b) pH 4 and 40 $^\circ\text{C}$, (c) pH 6.8 and 25 $^\circ\text{C}$, and (d) pH 9.6 and 25 $^\circ\text{C}$; (e) full spectrum of (a).

Figure 6.6 GPC traces for (a) CS, (b) *alkynyl*-CS, and (c) CS(-*g*-PDMAEMA)-*g*-PNIPAM in acetic acid buffers.

6.3.2 Thermo- and pH-responsive micellization of the graft CS terpolymer

The homopolymers of DMAEMA and NIPAM exhibit typical stimuli-responsive amphiphilicity. PDMAEMA is a weak polybase and its conjugated acid possesses a pK_a of about 7: it is soluble as a weak cationic polyelectrolyte due to the protonation of tertiary amine groups when $pH < 6$ while it becomes more hydrophobic and even water-insoluble at higher pH.^{31,49-50} PNIPAM dissolves in cold aqueous solution but becomes insoluble above 32 °C, which is ascribed to the lower critical solution temperature (LCST) phase transition behavior.⁵¹ The water solubility of PDMAEMA at alkaline pH is relatively high as compared to that of its counterpart, PDEAEMA, which serves as a hydrophobic core material for many pH-responsive micellar systems.^{28-32,52} As reported previously, CS-*g*-PDMAEMA demonstrated a

pH-induced association behavior at pH 5–6, because the CS had a $pK_a \sim 5.5$ and the deprotonated CS moieties formed the cores of micelles due to hydrophobic association and hydrogen-bonding interaction.^{6,9} In the present case, it is expected that the presence of pH-responsive CS/PDMAEMA segments and thermo-responsive PNIPAM side chains can make the CS(-g-PDMAEMA)-g-PNIPAM terpolymer exhibit a dually responsive feature. Two typical solution conditions [i.e. (a) pH 4 and 40 °C; (b) pH 10 and 25 °C] were selected to facilitate the micellization of the terpolymer through self-assembly. From the obvious characteristic bluish tinge of the colloidal dispersions under both conditions, the micellization processes could be persuasively evidenced. Subsequently, the critical micellization concentrations (cmc) of CS(-g-PDMAEMA)-g-PNIPAM under the two conditions were determined by measuring their surface tension variations with polymer concentration (Figure 6.7). The cmc values of the terpolymer under two conditions are both around 0.01 mg/mL, which are quite consistent with those of another reported Janus-type star copolymer, (PDEAEMA)-CD-(PNIPAM).³⁰

Figure 6.7 Surface tension versus concentration of CS(-g-PDMAEMA)-g-PNIPAM under two representative conditions: (a) pH 4 and 40 °C; (b) pH 10 and 25 °C.

The dual-responsive formation of different types of aggregates at different solutions has also been supported by the ^1H NMR results (Figure 6.5a-d).²⁹⁻³² At 25 °C and pH 4, all the segments of CS(-g-PDMAEMA)-g-PNIPAM are hydrophilic, so that the comb-like graft terpolymer molecularly dissolves in aqueous solution and the signals of the corresponding protons of PDMAEMA and PNIPAM are observed in Figure 6.5a. Upon heating the pH 4 solution to 40 °C, the signals characteristic of the PNIPAM side chains around $\delta = 1.0, 1.5, 1.9$ and 3.8 ppm (peaks f, h, i and g) thoroughly disappear, while the signals from PDMAEMA and CS are still visible (Figure 6.5b). The result demonstrate the insolubility of the PNIPAM segments. At the same time, the characteristic bluish tinge of the colloidal solution suggests the formation of micelles consisting of PNIPAM hydrophobic cores and well-dissolved

hybrid CS/PDMAEMA coronas. At 25 °C and pH 6.8, ^1H NMR resonance signal of CS at $\delta = 3.1$ ppm completely disappeared (see magnified Figure 6.5a&c), which represents the deprotonation and aggregation of CS segments. On the other hand, the characteristic signals of PDMAEMA at $\delta = 2.9, 3.4$ and 4.3 ppm (peaks a, b and c) also become weak and broad compared with those at pH 4, resulting from the decreased hydrophilicity of PDMAEMA which is attributed to the partial deprotonation of the tertiary amino groups.⁵³ Further addition of NaOD gave the final solution a pH of 9.6, and the characteristic NMR signals of PDMAEMA are intensively suppressed (shown in Figure 6.5d) due to the limited solubility of the fully neutralized PDMAEMA segments. Considering the appearance of a bluish tinge in the solution, another type of aggregates consisting of CS/PDMAEMA hydrophobic cores and PNIPAM hydrophilic coronas could be formed. The existence of a residual peak *a* from the segments reveals that this kind of micelle has a relatively less compact hydrophobic core than that PDEAEMA-cored micelles have, because PDMAEMA still has some water solubility at neutral or alkaline pH.^{29-31,52}

The following LLS studies provide more insights on the microstructures of the aggregates formed under different conditions. Figure 6.8a shows the pH dependence of intensity-average hydrodynamic radius (R_h) and the dimensionless ratio of gyration radius (R_g) to R_h for the CS(-*g*-PDMAEMA)-*g*-PNIPAM terpolymer at 25 °C. It is well known that the theoretical values of R_g/R_h for random coil, core-shell structure and hard sphere are 1.5, 0.9 and 0.77, respectively.⁵⁴ Below pH 4, terpolymer chains molecularly disperse in an acidic solution with an almost constant R_h (ca. 60 nm). The

ratio of R_g/R_h , ranging between 1.3 and 1.5 which are very close to the theoretical value for Gaussian chains, reveals that the polymer chains exist in random coil conformation owing to the strong electrostatic repulsion. Upon addition of NaOH, significant aggregation occurs over pH 5 to 7, as judged by the dramatic increase in R_h , corresponding to the major deprotonation of CS and PDMAEMA segments. Taking into account the association mechanism of the previously reported CS and PDMAEMA binary system, their pK_a difference leads to the formation of “onion-like” micelles consisting of CS core, PDMAEMA inner shell, and hydrophilic PNIPAM outer corona when $pH > 7$.^{9,31,55-56} It is notable that using the CS(-*g*-PDMAEMA)-*g*-PNIPAM terpolymer to replace the CS-*g*-PDMAEMA copolymer can increase the micelles’ stability in alkaline media due to the steric stabilization imparted by the hydrophilic PNIPAM segments. Above pH 8, the sizes of aggregates remain almost constant at $R_h \sim 150$ nm, and the R_g/R_h values are about 0.85–0.9, indicating the formation of a core-shell structure.^{9,57} The low polydispersity index ($\mu_2/I^2 \sim 0.1$) of aggregates (as shown in Figure 6.8b) shall be ascribed to the narrow dispersity of the terpolymer endowed by the ATRP-synthesized side chains. In addition, the change in zeta potential according to pH variation was summarized in Figure 6.8c. The zeta potential is more than +25 mV at $pH < 4$, and it decreases continuously to +3.8 mV when pH is increased to 8, which reflects the ongoing deprotonation process of the terpolymer. Finally, the averaged zeta potential approaches 0 mV at $pH > 9$ which reflects the loss of electrostatic charges on the surface of the micelles. However, the obtained transparent colloidal solution rather than cloudy precipitation further

confirms that the stability of micelles is rooted in the incorporation of the PNIPAM segments into the terpolymer.

Figure 6.8 (a) Variations of hydrodynamic radius (R_h) and the ratio of R_g/R_h as a function of pH at 25 °C for CS(-g-PDMAEMA)-g-PNIPAM in aqueous solution. (b) Intensity autocorrelation function and decay time distribution function for CS(-g-PDMAEMA)-g-PNIPAM at pH 10 and 25 °C. (c) Zeta potential of CS(-g-PDMAEMA)-g-PNIPAM terpolymer at different pH values.

Starting from the unimer state of the terpolymer in an aqueous solution at pH 4 and 25 °C, micelles consisting of PNIPAM cores and CS/PDMAEMA hybrid coronas can also be fabricated via heating. Figure 6.9 shows the temperature dependence of R_h and R_g/R_h for the CS(-g-PDMAEMA)-g-PNIPAM terpolymer at pH 4. Below 33 °C, the graft terpolymer molecularly dissolves with R_h varying between 62 and 66 nm. Aggregation starts to occur when temperature exceeds 33 °C, which is accompanied with a sharp increase in R_h . Above 40 °C, the size of the micelles remains almost constant at $D_h \sim 200$ nm. Synchronously, the value of R_g/R_h decreases from 1.5 to 0.9, demonstrating that the terpolymer has changed from random coils to core-shell structured micelles. It should be pointed out that the self-assembled aggregates under

both conditions are completely reversible as evidenced by LLS studies. The tuning of solution conditions can switch the terpolymer in aqueous solution to form microstructures between different types of aggregates and umimers.

Figure 6.9 Temperature-dependences of R_h and R_g/R_h in aqueous solution at pH 4 for CS(-g-PDMAEMA)-g-PNIPAM.

The actual morphologies of the aggregates formed under two representative conditions were further investigated by TEM micrographs shown in Figure 6.10. At pH 10 and 25 °C, spherical micelles with diameters ranging from 260 to 280 nm can be identified, which is in good agreement with the diameter determined by DLS considering the hydration effect. At the same time, the uneven darkness distribution in the particles corroborates the formation of core-shell structured micelles. At pH 4 and 40 °C, nearly spherical micelles consisting of PNIPAM hydrophobic cores and CS/PDMAEMA hydrophilic coronas can be observed and their mean diameter is

around 170 nm. On the basis of the results obtained by ^1H NMR, LLS and TEM, the conformational changes of CS(-*g*-PDMAEMA)-*g*-PNIPAM at different solution conditions were presented in Scheme 6.2.

Figure 6.10 TEM micrographs for self-assembled micelles obtained from 0.05 wt% CS(-*g*-PDMAEMA)-*g*-PNIPAM terpolymer solutions at: (a) pH 4 and 40 °C, and (b) pH 10 and 25 °C.

Scheme 6.2 Schematic description for the magic association behavior of the dual hydrophilic CS(-*g*-PDMAEMA)-*g*-PNIPAM terpolymer

6.4 Summary

To sum up, a novel dually stimuli-responsive graft terpolymer, CS(-g-PDMAEMA)-g-PNIPAM, based on the CS derivative, has been synthesized via the combination of ATRP and click reaction. The utilization of ATRP guaranteed the narrow molecular weight distribution of the side chains, which further brought on a low polydispersity to the terpolymer. The click reaction taking place in mild and homogeneous solution was shown to be an efficient coupling method to obtain a high degree of grafting. The graft terpolymer could aggregate into three-layer “onion-like” micelles at 25 °C when pH was above 7. Moreover, spherical micelles with PNIPAM cores were obtained around 40 °C in acidic solution (pH ~ 4). Most importantly, switching between different types of aggregates and unimers could be reversibly achieved by properly tuning pH and temperature of the aqueous solutions. This work represents the first such example of a dual hydrophilic CS copolymer showing the magic micellization behavior.

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Chapter 7

Thermo-Responsive Transfection of DNA Complexes with Well-Defined Chitosan Terpolymers

7.1 Introduction

Non-viral gene carriers have drawn more and more attention for their potential applications in the field of gene therapy. Despite considerable effort having been devoted to the development of both natural and synthetic agents for gene delivery, the efficiency of gene transfection with non-viral carriers has yet to achieve a competitive level.¹⁻² Polymer/DNA complexes must overcome a series of obstacles in the transfection process, including traversing the cellular membrane, escaping from the endosome, and entering the nucleus. In the first two stages, the complex fabricated from a carrier and DNA should be stable and compact to protect the plasmid from being degraded by enzymes. But when DNA is transported into the nucleus, the complex should be loose enough to allow DNA release. Obviously, traditional cationic polymers cannot satisfy these contradictory requirements at the same time.

One of the promising routes to modulate or switch the affinity of a carrier to DNA relies on the use of responsive or “smart” polymers that are capable of conformational or phase changes under different regimes of pH and temperature. One of the most widely used polymer, poly(*N*-isopropylacrylamide) (PNIPAM), is a water-soluble polymer that undergoes a coil-to-globule phase transition at 32 °C (lower critical solution temperature, LCST).³ The pioneering study carried out by Hennink et

al.⁴ suggested the use of poly(*N*-isopropylacrylamide)-*co*-poly((2-dimethylamino)ethyl methacrylate) (PNIPAM-*co*-PDMAEMA) as pH- and thermo-dependent carriers for gene therapy. Thereafter, Okano and coworkers introduced hydrophobic comonomers (i.e. butylmethacrylate (BMA)) into PNIPAM-*co*-PDMAEMA copolymers and reported enhanced DNA binding, release and transfection as a consequence.⁵⁻⁷ Several works were published recently describing the application of variant copolymers of poly(ethylenimine) (PEI) and PNIPAM for gene delivery *in vitro*.⁸⁻¹² These copolymer/DNA complexes displayed variations in physicochemical behaviors above and below the LCST, which further affected their cellular uptake and transfection activity. Some cationic peptides, such as poly(L-lysine) (PLL) and poly(L-arginine) (PArg), were also conjugated with PNIPAM to fabricate potential thermo-responsive gene carriers.¹³⁻¹⁴

In recent years, grafting of PNIPAM as side chains onto chitosan (CS) constitutes a fast-growing area of research because it makes it possible to achieve both temperature and pH responsive materials.¹⁵⁻¹⁸ For example, a comb-like CS-*g*-PNIPAM copolymer synthesized by Chen and Cheng was utilized as a cell-carrier for transplantation of chondrocytes and meniscus cells for tissue engineering of cartilage.¹⁵ Initially, Sun et al. prepared a carboxyl-terminated NIPAM/vinyl laurate copolymer and coupled it with CS, and the resulting graft terpolymer displayed a LCST of about 26 °C.¹⁹ The dissociation of the modified CS/DNA complexes could be tuned by varying the temperature: at 37 °C, the collapse of PNIPAM is favorable to form the compact nanoparticles; whereas at 20 °C, the hydrophilic and extended

PNIPAM chains facilitate the unpacking of DNA from the complexes. Driven by this concept, Mao et al. successfully synthesized trimethyl CS (TMC)-*g*-PNIPAM, which was demonstrated to be an effective thermo-responsive gene carrier with a high transfection efficiency and minimal cytotoxicity.²⁰ In Chapter 6, we synthesized a novel dually stimuli-responsive graft terpolymer, CS(-*g*-PDMAEMA)-*g*-PNIPAM, using a facile “click” technique. For this kind of terpolymer, different types of aggregates can be achieved by properly altering pH and temperature of the aqueous environment. In this chapter, the thermo-dependency of serial graft CS terpolymers/DNA complexes shall be elucidated in terms of the physicochemical properties and gene transfection efficiency, in order to demonstrate their great potential as novel gene carriers.

7.2 Experimental

7.2.1 Synthesis of graft CS terpolymers

The experimental details for the synthesis of dually stimuli-responsive graft CS terpolymer (denoted as TCS), CS(-*g*-PDMAEMA)-*g*-PNIPAM, have been described in Section 6.2. A series of new cationic polymers composed of CS backbones, PDMAEMA and PNIPAM side chains have been developed successfully. Compared with the final product reported in Chapter 6, a slight difference lies on the length of the PDMAEMA side chains, since the cytotoxicity of polycations is known to increase with molecular weight. Based on the recent findings by Ping et al., the CS copolymer conjugated with shortest PDMAEMA side chains (ca. 15 repeating units) exhibited the minimal cytotoxicity while maintaining a good gene transfection

capacity.²¹ Hence, shorter PDMAEMA side chains (16 repeating units) are used in this chapter by adjusting the feed ratio between ATRP initiator and monomer.

7.2.2 Characterization

The protocol and instrumentation used in the ¹H NMR and GPC experiments have been presented in Chapters 5 and 6. The morphology of the TCS/DNA complexes at different N/P ratios was observed using transmission electron microscopy (TEM, JEOL JEM-2010) operating at an accelerating voltage of 200 kV. In order to observe the thermal influence on the morphology of particles, the complexes were firstly prepared at 20 °C and incubated for 30 min. Then the solution temperature was set to 20 or 37 °C respectively, and the solution was dripped on the copper grids and freeze-dried.

Dynamic light scattering (DLS) and zeta potential analyses were performed using a Malvern Zetasizer Nano ZS instrument with a laser light wavelength of 633 nm at a 173° scattering angle, and all data were averaged over three measurements. To determine the LCST of a TCS solution, TCS was firstly dissolved in acetic acid buffer (pH 5.4) to form a 0.03 wt% solution and filtered through a 0.8 µm filter to remove dust and any other insoluble components.¹⁹ The scattering light intensity as a function of temperature was measured, and the inflection point was defined as the LCST of the terpolymer. For DLS of TCS/DNA, 100 µL of complexes containing 3 µg of pDNA were prepared at various N/P ratios. The mixture was vortexed for 6 s, incubated at 20 °C for 30 min and diluted to 1 mL by 15 mM of KCl solution at the time of measurement. The zeta potential measurements were carried out using a capillary cell

under the Smoluchowski approximation, and 20 to 100 runs were automatically chosen for a good reproducibility.

7.2.3 Cell culture and cell viability

COS7 and HeLa cell lines were purchased from ATCC (Rockville, MD). The cells were cultured in a DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mg penicillin, and 100 µg/mL streptomycin at 37 °C, 5% CO₂, and 95% relative humidity. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazan. In detail, 100 µL of cells were seeded into 96-well plates at a density of 1×10^5 cells/mL. After incubation for 24 h, the culture media were replaced with 100 µL of fresh media containing the terpolymer at different concentrations for further 20 h. Next, 10 µL MTT (5 mg/mL in PBS) was added to each well for 4 h. The supernatant was removed by an aspirator after that, and the formazan crystals were dissolved in DMSO (100 µL/well), followed by shaking for 5 min. The absorbance was read using a microplate reader (Spectra Plus, TECAN) at a wavelength of 570 nm. The cell viability (%) relative to control cells was calculated from $A_{\text{test}}/A_{\text{control}} \times 100\%$, where A_{test} and A_{control} are the absorbance values of the wells (with the composites) and control wells (without the composites), respectively. For each sample, the final absorbance was the average of those measured from six wells in parallel.

7.2.4 Plasmid DNA preparation and gel retardation assay

The plasmid DNA used as reporter gene was pRL-CMV (Promega, USA), which encoded *Renilla* luciferase originally cloned from the marine organism *Renilla reniformis*. The plasmid DNA was amplified in *E.Coli* and purified by using a commercial kit according to the supplier's protocol (Qiagene, Hilden, Germany). The quantity and quality of the purified pDNA were assessed by optical density at 260 and 280 nm. The purified pDNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and kept in aliquots at a concentration of 0.50 mg/mL.

The CS terpolymers' capability to inhibit pDNA migration was examined through gel electrophoresis experiments. The TCS/DNA complexes were prepared by adding 10 μ L of a TCS solution (pH 5.4) dropwise to an equal volume of the plasmid solution containing 0.4 μ g DNA, followed by vortexing for 6 s and incubated at room temperature for 30 min. After mixing 5 μ L of 5 $\times$ loading buffer with complex solutions, 20 μ L of complex solution was analyzed on 0.9% agarose gel containing 0.5 μ g/mL ethidium bromide. Gel electrophoresis was carried out in 1 $\times$ Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA) at 100 V for 30 min at different temperatures in a Sub-Cell system (Bio-Rad Laboratories, CA). DNA bands were visualized by a UV lamp using a GelDoc system (Synoptics Ltd., UK).

7.2.5 *In vitro* gene transfection assays

For *in vitro* transfection studies using plasmid pRL-CMV, cells were seeded into a 24-well plate at a density of 5×10^4 cells per well in 0.5 mL of DMEM containing antibiotics for 24 h prior to transfection. At the time of transfection, the

cells had grown to approximately 70–80% confluence, and the medium in each well was replaced with 0.3 mL of serum-rich medium. 20 μ L of complexes containing 1 μ g pDNA were added to each well and incubated with the cells at 37 °C for 4 h. Then the medium was replaced with 0.5 mL of fresh DMEM and cells were further incubated under two different conditions: (i) 44 h at 37 °C and (ii) 18 h at 37 °C + 2 h at 20 °C + 24 h at 37 °C. Finally, cells were washed by PBS and lysed with the reporter lysis buffer. The luciferase assay was carried out according to the supplier's protocol (Promega, USA), and luciferase activity was measured on a single-well luminometer (Berthold lumat LB9507, Germany) for 10 s. The relative light units (RLU) were normalized against protein concentration in the cell extracts, which was measured using the bicinchoninic acid assay kit (Biorad, CA, USA). Absorption was measured on a microplate reader (Spectra Plus, TECAN) at 570 nm and compared to a standard curve calibrated with bovine serum albumin (BSA) samples of known concentration. Results are expressed as relative light units per milligram of cell protein lysate (RLU/mg protein).

Besides, plasmid pEGFP-N1 (Clontech laboratories Inc. USA) encoding a red-shift variant of wild type green fluorescence protein (GFP) was also applied to verify the gene transfection. Briefly, cells were seed into a 12-well plate at a density of 1×10^5 cells/well in 1 mL of complete DMEM. After 24 h, 600 μ L of reduced serum Opti-MEM, to which 40 μ L complexes containing 2 μ g of pDNA was added, was used to replace the former culture medium. After 4 h, the transfection medium was removed and the cells continued to be incubated in serum-containing medium according to the

constant and variable temperature protocols. At the end of transfection, the cells were washed with PBS buffer twice and observed under a laser scanning confocal microscope (LSM 410, Carl Zeiss, USA). The green fluorescence was excited at 488 nm and its emission was collected using a 515 nm filter.

7.3 Results and discussion

7.3.1 Characterization of TCS

Well-defined comb-shaped terpolymers consisting of CS, PDMAEAM₁₆ and PNIPAM₃₂ side chains were synthesized via ATRP and click techniques. Table 7.1 summarizes the chemical composition of TCS polymers. TCS with different side chain components can be achieved by using different *alkynyl*-CS precursors and varying the feed ratios of PDMAEMA to PNIPAM during the click reactions. Figure 7.1 shows the typical ¹H NMR spectrum of TCS1, from which the average numbers of PDMAEMA and PNIPAM chains per CS backbone were estimated. Similar to the former discussion, the theoretical M_n can be determined by assuming that no degradation occurred during the click reaction. In fact, GPC results show that the molecular weight of the terpolymers increased steadily from 24000 to 51000 g/mol, whereas M_w/M_n decreased from 1.52 to 1.35 (by benefiting from the incorporation of increasing number of side chains having narrow molecular mass distribution). The molecular weight of TCS estimated by ¹H NMR is higher than that obtained from GPC measurements (Table 7.1). The difference in hydrodynamic volumes between comb-structured TCS and linear polysaccharide standard should be responsible for the GPC underestimated M_n results.

Figure 7.1 ^1H NMR spectrum recorded in $\text{D}_2\text{O}/\text{DCl}$ for TCS1.

Table 7.1 Composition and molecular characteristics of TCS carriers

Sample	Weight ratio in TCS (%) ^a			$M_{n,\text{theo}}$ ^a (g/mol)	$M_{n,\text{GPC}}$ ^b (g/mol)	M_w/M_n ^b	LCST ^c (°C)
	PDMAEMA	PNIPAM	CS				
TCS1	37	30	33	34000	24000	1.52	34.4
TCS2	43	34	23	52000	37000	1.42	34.7
TCS3	54	28	18	65000	41000	1.40	35.1
TCS4	29	57	14	96000	51000	1.35	32.5

^a Determined by ^1H NMR.

^b Measured by GPC, pullulan standards.

^c Determined by DLS.

The temperature-induced phase transition in aqueous solution is rather typical and well established for PNIPAM and its copolymers. To determine the LCST of TCS terpolymers in PBS buffer, we measured the light scattering intensity as a function of temperature by DLS. Depending on the PNIPAM weight gradients of TCS1–TCS4 samples, the LCST varied from 32.5 to 35.1 °C as listed in Table 7.1. At a temperature below the LCST, no aggregates were detected and the light scattering intensity was low, implying that terpolymers were molecularly dispersed. Above the LCST, TCS formed aggregates with hydrophobic PNIPAM cores and partially protonated PDMAEMA and CS corona. It is worth mentioning that the LCST in PBS buffer should be lower than in pure water and acid buffer, as a result of the salt out and pH effect, respectively.^{20,22}

As mentioned earlier, cytotoxicity is one of the most important criteria in selecting polymeric gene carriers for clinical gene therapy. Figure 7.2 demonstrates the *in vitro* MTT assay results for the cytotoxicity of TCS1–4, PDMAEMA, and PEI in COS7 and HeLa cell lines. For both cells, all the cationic polymers exhibited a dose-dependent effect on cytotoxicity. However, TCS1–4 showed much lower toxicity than PDMAEMA and PEI, which can be judged from the slopes of their cell viability curves. It is generally accepted that a high concentration of amino groups plays an important role in causing high cytotoxicity.²¹ Among the TCS terpolymers, TCS2 and TCS3 exhibit slightly higher cytotoxicity (especially at high polymer concentration) than others, probably due to the higher density of tertiary amino groups from PDMAEMA. Therefore, the introduction of the nontoxic CS and PNIPAM chains not

only decreases the relative concentration of amino groups, but also imparts biocompatible characteristics to the TCS carriers. It is worth noting that the cytotoxicity of all cationic polymers is also cell-type dependent. For TCS carriers, polymer concentration displays a more profound and toxic impact on the HeLa cells compared to that on COS7 cells (as shown in Figure 7.1).

Figure 7.2 Cell viability of TCS polymers at different concentrations in (a) COS7 and (b) HeLa cell lines, where PEI (25 kDa) and PDMAEMA were used as controls.

7.3.2 Physicochemical properties of TCS/DNA complexes

DNA condensation induced by carriers into nanoparticles is a prerequisite for successful cellular uptake and transfection. Herein, the ability of TCS carriers to encapsulate pDNA into particulate structures was verified by agarose gel electrophoresis, DLS, zeta potential, as well as TEM. As shown in Figure 7.3, the weight ratios (TCS to DNA) of 2–3 are sufficient for completely inhibiting plasmid migration. Considering the difference in molar ratio of PDMAEMA and CS among terpolymers, the weight ratios could be converted into N/P ratios. Thus, full retardation of the DNA was achieved at the N/P ratios of about 3.8, 2.5, 2.8 and 1.7 for TCS1–4, respectively. TCS1 was found to be less efficient to condense DNA in comparison with TCS2 and TCS3, which should be ascribed to the lower DMAEMA concentration which induced the lower cationic charge density. As depicted previously, the pK_a of DMAEMA is higher than that of CS, implying that the electrostatic contribution mainly comes from DMAEMA. Nevertheless, even though the polycation concentration of TCS4 is less than that of others, it still can retard DNA migration efficiently. The enhanced condensation capability of TCS4 may benefit from the larger molecular weight, thus leading to more significant intermolecular interactions.

Figure 7.3 Electrophoretic mobility of pDNA in the complexes of the cationic polymers (a) TCS1, (b) TCS2, (c) TCS3, and (d)TCS4 at various polymer/plasmid weight ratios.

Particle size (Figure 7.4) and zeta potential (Figure 7.5) of TCS/DNA complexes at various N/P ratios were also investigated, since they are important factors for determining the rate of cellular uptake. Generally speaking, all cationic terpolymers can efficiently compact pDNA into nanoparticles in the diameter range of 50–150 nm with a narrow PDI at high N/P ratios (≥ 8). Within this size range, TCS/DNA complexes are able to undergo endocytosis readily. Meanwhile, zeta potential is an essential factor affecting the stability and cellular uptake of nanoparticles by indicating their surface charges. Positively charged surfaces of nanoparticles are believed to bind to negatively charged cell membranes, further facilitating the non-specific endocytosis. As shown in Figure 7.5, the zeta potential of

TCS/DNA complexes increases dramatically as the N/P ratio increases from 1 to 4 and stabilizes at N/P ratio of 8 or higher. On the other hand, the presence of sufficient amount of positive/negative charges on the complex surface is the major driving force to prevent aggregation via Coulombic repulsion between particles. This point helps to explain the zigzag particle size change observed for TCS1 and TCS4 complexes at low N/P ratios (≤ 4 in Figure 7.4). Taking TCS1/DNA for instance, a very large particle size was obtained at N/P = 4 due to its zeta potential being close to 0 mV. Therefore, as Erbacher et al. summarized, increasing the N/P ratio decreases the size of the complex, except for the conditions where neutral particles are formed.²³

Figure 7.4 Particle size of the TCS/DNA complex at various N/P ratios.

Figure 7.5 Zeta potential of the TCS/DNA complex at various N/P ratios.

Figure 7.6 shows the representative TEM images of the TCS4/DNA complexes at different N/P ratios and temperatures. At an N/P ratio of 2, large, loose and irregular particles can be observed, indicating that the pDNA was not tightly condensed. At an N/P ratio of 12, nearly spherical and core-shell structured nanoparticles are clearly demonstrated (Figure 7.6 b&c), and the size distribution is consistent with that obtained from DLS measurements. The more compact core of nanoparticles consists of CS and PDMAEMA/plasmid complexes, while the corona is composed of hydrophilic and neutral PNIPAM chains. In order to confirm the thermo-responsive behavior of TCS/DNA complexes, the microstructure of TCS4/DNA above LCST was also studied. Compared with the remarkable core-shell characteristic of nanoparticles formed at 20 °C, more uniform morphology of particles can be unveiled at 37 °C (Figure 7.6 d&e). The shrinkage of outer shell of complexes can be attributed

to the collapse of the PNIPAM chains at temperature above the LCST. Agarose gel electrophoresis experiment for TCS4/DNA complexes at lower weight ratios was also carried out at 20 °C and 37 °C (Figure 7.7). A noticeable difference in the retardation bands can be discerned when the temperature below or above the LCST of TCS4. At 37 °C, a fully formed complex is observed at a mass ratio of 0.6/1. This further confirms that the collapsed PNIPAM chains shield more pDNA and charges, which leads to complex formation at a lower carrier ratio relative to 20 °C condition.

Figure 7.6 Representative TEM micrographs of (a) TCS4/DNA (N/P = 2, 20 °C), (b) TCS4/DNA (N/P = 12, 20 °C) and (d) TCS4/DNA (N/P = 12, 37 °C). Magnified morphologies of nanoparticles in (b) and (d) are presented in (c) and (e) correspondingly.

Figure 7.7 Electrophoresis of TCS4/DNA complexes on agarose gels at (a) 20 °C and (b) 37 °C with various weight ratios.

7.3.3 Thermo-responsive *in vitro* gene transfection

The *in vitro* transfection efficiency of TCS/DNA complexes was firstly assessed using luciferase as a marker gene in COS7 and HeLa cell lines. We adopted two protocols to examine the thermal regulation of transfected gene expression: (i) constant temperature approach (i.e. the culture temperature is kept at 37 °C); (ii) variable temperature approach (i.e. the transfected cells are incubated at 37 °C for 18 h, 20 °C for 2 h, and 37 °C for 24 h). Figure 7.8 and Figure 7.9 display the gene transfection efficiency mediated by TCS carriers in comparison with those of PDMAEMA and PEI (25 kDa) controls of COS7 and HeLa cells under two different protocols. In general, gene transfection efficiency is largely dependent on the cell types and N/P ratios of the complexes. In both cell lines, the transfection efficiency firstly increases at lower N/P ratios and then decreases after reaching a certain N/P ratio. The optimal N/P ratio of gene transfection for TCS1, TCS2, TCS3, TCS4, are 25, 20, 20, and 25 respectively, which are higher than those (10~15) of PDMAEMA and PEI.²¹ At lower N/P ratios, pDNA cannot be condensed effectively by the carriers,

and the resultant loose polymer/DNA complexes do not enter the cells easily. At higher N/P ratios, the presence of an increasing amount of cationic polymers induces increasing cytotoxicity and results in a reduction in the transfection efficiency. But in most cases, the transfection efficiencies of TCS2 and TCS3 are higher than those of TCS1 and TCS4. Considering that the side chains used for all the TCS terpolymers are the same, the difference in transfection capability should be ascribed to the chemical components, especially the inner ratios between PDMAEMA and CS segments. The larger content of PDMAEMA in a terpolymer can increase the binding ability and complexing stability, thus leading to a higher transfection efficiency. In comparison with the homopolymeric PDMAEMA control, the TCS2 and TCS3 carriers exhibit superior gene transfection efficiencies at optimal N/P ratios. The enhanced transfection ability is probably attributed to the biocompatibility endowed by CS and PNIPAM incorporation. Another possible reason is that the unique comb-like architecture could improve the interaction of the cationic PDMAEMA with pDNA or cellular membranes.^{21,24}

Thermo-responsive transfection efficiency should be attributed to the change of affinity between a TCS carrier and pDNA. As mentioned above, the phase transition of TCS plays an important role in the formation and dissociation of the complexes according to the change of temperature. At body temperature (37 °C), the PNIPAM chains become dehydrated and collapsed to form compacted TCS/DNA complexes, which could be internalized easily via endocytosis and protect pDNA from degradation by DNase. After 18 h incubation at 37 °C, the incubation

temperature was lowered to 20 °C for 2 h. Below the LCST, the PNIPAM chains are hydrated and become loose and extended, which can facilitate complex dissociation and gene exposure to result in increased gene expression. In order to clearly show the influence of temperature on the gene transfection, TCS3 and TCS4 were chosen as representatives to compare their transfection efficiencies for COS7 cells under two thermal protocols. As illustrated in Figure 7.10a, the transfection efficiency of TCS3/DNA using protocol (i) is slightly higher than those of using protocol (ii) at all N/P ratios. However, TCS4 exhibits the typical and obvious thermo-responsive behavior in gene transfection, and the luciferase activities of TCS4/DNA under the variable temperature condition increased 1–4 fold relative to that obtained under a constant temperature condition (shown in Figure 7.10b). This difference is of statistical significance. It is necessary to point out that temporarily lowering the incubation temperature may deteriorate transfection efficiency of PDMAEMA and PEI controls due to declined cell vitality.^{14,19-20} Such a “cold shock” mechanism also helps to explain why TCS3’s transfection efficiency shrinks after 2 h of incubation at 20 °C, although the lower temperature promotes the unpacking of pDNA from the complexes. Therefore, the variable temperature modulation cannot guarantee the improvement of gene expression, and there exists a competitive balance between the suppressed cell vitality and strengthened gene release. At the same time, it is worth emphasizing that a higher PNIPAM composition is also crucial to impart TCS more effective thermal control to transfected gene expression. Within the family of TCS, only the cationic polymer with a molar ratio of NIPAM/DMAEMA exceeding 2 (i.e.

TCS4) can show a distinctive thermo-responsive gene transfection property, which is quite consistent with the conclusion from the previous reports.^{5,7} What is more, by varying the feed ratios of the side chains during the click reaction, the molar ratio of NIPAM/DMAEMA can be easily adjusted to result in various carriers according to real applications.

In an attempt to confirm that the gene delivered to cultured cells are expressed in a thermo-responsive manner, direct visualization of gene expression of GFP in COS7 cells was also performed by fluorescence microscopy. Plasmid pEGFP-N1 encoding GFP was delivered by TCS4 (N/P = 25) through two temperature routes to examine the GFP expression, and the representative images are shown in Figure 7.11. Obviously, GFP is successfully expressed in COS7, and the variable temperature protocol results in stronger fluorescence signal than the constant temperature one. This conclusion is in good agreement with the luciferase reporter system.

Figure 7.8 *In vitro* gene transfection efficiency of TCS/DNA complexes (against N/P ratio) in comparison with those mediated by PEI (25 kDa) (N/P ratio = 10) and PDMAEMA (N/P ratio = 10) in COS7 cell line under (a) constant temperature and (b) variable temperature conditions.

Figure 7.9 *In vitro* gene transfection efficiency of TCS/DNA complexes (against N/P ratio) in comparison with those mediated by PEI (25 kDa) (N/P ratio = 10) and PDMAEMA (N/P ratio = 10) in HeLa cell line under (a) constant temperature and (b) variable temperature conditions.

Figure 7.10 Thermo-responsive transfection efficiency of (a) TCS3/DNA and (b) TCS4/DNA complexes (against N/P ratio) in comparison with those mediated by PEI (25 kDa) (N/P ratio = 10) and PDMAEMA (N/P ratio = 10) in COS7 cell line. Route (i): 48 h at 37 °C; (ii) 22 h at 37 °C + 2 h at 20 °C + 24 h at 37 °C.

Figure 7.11 Representative confocal microscopy images of COS7 cells transfected with pEGFP-N1 plasmid mediated by TCS4 (N/P = 25) under (a) constant temperature and (b) variable temperature conditions.

7.4 Summary

A series of well-defined graft CS terpolymers (TCS) composed of CS backbones, PDMAEMA and PNIPAM side chains have been developed as gene carriers via the ATRP and click chemistry methods. These TCS carriers demonstrate much lower cytotoxicity compared with the PDMAEMA and PEI controls, and high capability to complex pDNA into nanoparticles for gene transfection. In addition, the complexation between plasmid and cationic carriers could be tuned by changing the surrounding temperature. At temperature above the LCST, tight and stable complexes are formed due to the collapse of the PNIPAM chains in the TCS. Reducing temperature below LCST facilitates the dissociation of TCS/DNA complexes and exposure of pDNA. Thus, the level of gene expression can be thermally modulated and increased by lowering the incubation temperature to 20 °C after the internalization of TCS4/DNA complexes in COS7 and HeLa cells. The thermo-responsive gene

transfection efficiency is also influenced by the PNIPAM weight ratio in TCS carriers.

In conclusion, the thermo-sensitive CS(-g-PDMAEMA)-g-PNIPAM terpolymers are a new class of intelligent non-viral carriers with minimal cytotoxicity and good performance for gene transfer, which have a great potential in practical applications.

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Chapter 8

Chitosan-Functionalized Graphene Oxide as a Nanocarrier for Drug and Gene Delivery

8.1 Introduction

Graphene oxide (GO), a novel two-dimensional nanomaterial prepared from natural graphite, has recently attracted significant attention as a precursor of chemically converted graphene.¹⁻³ GO can be readily exfoliated into monolayer sheets to yield stable suspensions in water because of the hydrophilic oxygenated functional groups on the basal planes and edges.⁴⁻⁵ These groups enable GO to be functionalized through covalent and non-covalent approaches, hence making it a building block for synthesizing versatile functional materials.⁶⁻⁸ Specifically, covalently grafting polymers onto GO sheets is an efficient way to improve the properties of graphene such as solubility,⁹ interfacial interactivity with a target matrix,¹⁰ and electronic properties.¹¹ For example, Yang et al. successfully grafted the poly-(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) chains on the surface of GO nanosheets by *in situ* atom transfer radical polymerization (ATRP).¹² After grafting of PDMAEMA, the dispersity of GO sheets in a solvent was improved significantly. This surface-initiated polymerization tactic was also applied by many researchers to graft various polymers onto graphene sheets and then fabricate graphene-based nanocomposites.¹³⁻¹⁵ Despite the steric hindrance and diffusion restrictions, directly coupling the functional groups on polymer chains with hydroxyls, epoxides or

carboxylic acids on GO sheets still remains a facile and promising way to functionalize graphene. Several water-soluble polymer grafted graphene nanocomposites have been synthesized using different coupling techniques. Veca et al.⁹ and Salavagione et al.¹⁶ independently functionalized graphene sheets with poly (vinyl alcohol)(PVA) via an etherification reaction. The PVA-functionalized graphene sheets were readily soluble in aqueous and polar organic solvents to form stable solution-like dispersions.

In recent years, there is a surge of interest in exploring graphene and its derivatives for medical and biological applications (e.g. drug loading and delivery) due to its biocompatibility, unique conjugated structure, large surface area and relatively low cost.¹⁷⁻²⁰ Dai's group initially developed polyethylene glycol (PEG) functionalized nanoscale graphene oxide (NGO) as a nanocarrier to load anticancer drugs via noncovalent physisorption and evaluated its *in vitro* cellular uptake capacity.²⁰ Subsequently, it was testified that PEGylated nanographene sheets (NGS) themselves also exhibited ultrahigh *in vivo* tumor uptake and efficient photothermal therapy properties in mice.²¹ Yang and colleagues investigated the loading and release behaviors of doxorubicin hydrochloride (DXR) on GO, and they found that the weight ratio of the loaded drug to the GO carrier could reach 200%.²² Inspired by these findings, Zhang et al. functionalized NGO with sulfonic acid and folic acid (FA) groups, which rendered it physiological stability and specific cells targeting property correspondingly.²³ Furthermore, controlled loading of two anticancer drugs, doxorubicin (DOX) and camptothecin (CPT), onto the FA-conjugated NGO (FA-

NGO) via π - π stacking and hydrophobic interactions was studied. The co-delivery of two drugs by FA-NGO revealed specific targeting to MCF-7 cells, and remarkably high cytotoxicity compared to NGO loaded with either DOX or CPT only.

Chitosan (CS) has found widespread bioapplications in drug and gene delivery, tissue engineering, and as a pharmaceutical ingredient, because of its good biocompatibility, biodegradability, low immunogenicity and antibacterial property.²⁴⁻²⁷ In addition, CS is a stimulus-responsive polymer with a solubility that can be adjusted reversibly by changing the pH.²⁸⁻²⁹ Dispersing GO sheets into CS aqueous solutions under proper pH conditions, we have fabricated uniform CS films which were significantly reinforced by the parallel alignment of GO nanofillers.³⁰ Coincidentally, a quite similar work about CS/GO nanocomposites was also reported on line during our submission of that manuscript.³¹ It is interesting to note that another research group just prepared a homogeneous graphene aqueous suspension by chemical reduction of GO in the presence of CS.³² They demonstrated that the graphene in the as-prepared suspension can be switched reversibly between a well dispersed and a more aggregated state with pH as a stimulus. In the above-mentioned literature, noncovalent interactions between GO and CS played an important role in the initial formation of homogeneous graphene suspensions. However, the introduction of covalent conjugation between GO and CS to fabricate a new kind of nanocomposite, whose properties are expected to incorporate their individual merits, has not been conducted so far.

In the present chapter, we have grafted CS chains onto the GO surface using an amide linkage. The success of the functionalization was verified by ^1H NMR, FTIR, XPS, TGA, potentiometric titration, and AFM. The CS modified GO (GO-CS) sheets display good solubility in acidic and neutral aqueous solutions and formed stable dispersions. Due to the conjugated structure of graphene basal planes, GO-CS can adsorb aromatic, water insoluble drugs via van der Waals interaction. The application of GO-CS in loading and delivering a representative anticancer drug (CPT) was investigated, and the MTT assay uncovered that GO-CS-CPT could afford a high potency to kill cancer cells *in vitro*. Meanwhile, CS is emerging as an ideal candidate for gene delivery with a reasonable transfection efficiency combined with a minimal level of cytotoxicity. GO-CS sheets could effectively encapsulate plasmid DNA to form compact complexes, which exhibited relatively good transfection efficiency in HeLa cells at certain N/P ratios. It is believed that, with these new functionalities, GO-CS presents a great potential in biological applications for drug and gene delivery.

8.2 Experimental

8.2.1 Materials

Graphite with an average particle size of 100 μm was obtained from Fluka. Chitosan ($M_n = 3$ kDa) with 90% degree of deacetylation (DD, determined by ^1H NMR) was provided by Haidebei Marline Bioengineering Co., Ltd., China. 2-(*N*-morpholino)ethanesulfonic acid (MES, 99%), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl, 99%), *N*-hydroxysuccinimide (NHS, 97%) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT,

98%) were obtained from Aldrich and directly used. Camptothecin (CPT) was purchased from Guanyu Bio Inc., China. Dulbecco's modified eagle medium (DMEM) was purchased from Gibco BRL (Gaithersburg, MD). Other reagents mentioned in this chapter were purchased from Aldrich and used as received.

8.2.2 Preparation of GO

First, graphite powder (3 g) was charged into an 80 °C solution consisting of concentrated H_2SO_4 (97 %, 12 mL), $\text{K}_2\text{S}_2\text{O}_8$ (2.5 g), and P_2O_5 (2.5 g). The mixture was kept at 80 °C for 6 h using a hotplate. Successively, the mixture was cooled to room temperature and diluted with 0.5 L of deionized (DI) water and left to stand overnight. Then the mixture was filtered and washed with DI water using a 0.2 μm Nylon filter to remove residual acid. The product was dried under ambient condition overnight. This pre-oxidized graphite was then subjected to oxidation by the Hummers method.³³ Briefly, 1 g of pretreated graphite and 0.5 g of NaNO_3 were placed in a flask. Then, 25 mL of H_2SO_4 was added with stirring in an ice-water bath, and 3 g of KMnO_4 were slowly added over about 1 h. Stirring was continued for 2 h in the ice-water bath. After the mixture was stirred vigorously at room temperature for 2 days, 100 mL of 5 wt% H_2SO_4 aqueous solution was added over about 1 h with stirring, and the temperature was kept at 98 °C. The resultant mixture was further stirred at 98 °C for 2 h. The temperature was reduced to 60 °C, 3 mL of H_2O_2 (30 wt% aqueous solution) was added, and the mixture was stirred at room temperature for 2 h. The oxidation product was purified by rinsing with a 10% HCl solution,

repeatedly washing with copious amounts of water, and filtering through a 0.2 μm Nylon membrane.

8.2.3 Preparation of chitosan grafted GO (GO-CS) sheets

The GO precursor has been synthesized using a modified Hummers method from natural graphite powder.³³⁻³⁴ Then the nanocarrier for drug and gene delivery, GO-CS, was prepared by the amidation of GO with CS in the presence of EDC and NHS. In a typical procedure, CS (0.5 g, 2.77 mmol) and GO (0.1 g, 0.17 mmol) were firstly dispersed in 50 mL of MES buffer (0.1 M, pH adjusted to 5) and sonicated for 1 h to get a homogeneous colloidal suspension. Being protected by argon, EDC (0.652g, 3.4 mmol) and NHS (0.782 g, 6.8 mmol) were gradually charged into the flask within 20 min. The reaction was conducted at room temperature under bath sonication for 6 h and stirring for another 16 h. After the reaction was terminated, the suspension was filtered over a 0.2 μm Nylon microporous membrane and thoroughly washed with a large amount of acetic acid solution (0.1 M) to remove unreacted CS. Thereafter, the collected solid was redispersed and dialyzed (MWCO = 8 kDa) against DI water for 3 days at 4 °C. The final product was lyophilized to give a yield of about 95% (0.29 g).

8.2.4 Loading of CPT on GO-CS and *in vitro* release

Briefly, 10 mL of 0.5 mg/mL GO-CS in DI water was mixed with 1.0 mL of 8.6 mM CPT DMSO solution and stirred overnight at room temperature. Excess CPT, which precipitated as a solid, was removed by centrifugation. The supernatant was filtered through a 0.8 μm filter to fully remove any solid. The obtained solution was then dialyzed (MWCO = 12 kDa) against distilled water for 3 days to remove the

small amount of solubilized free CPT and DMSO. The formed GO-CS-CPT complex solution was preserved in darkness at 4 °C.

To examine the drug release from the GO-CS, 2 mL prepared GO-CS-CPT was loaded into an inner dialysis tube (MWCO = 10 kDa) and dialyzed against 15 mL PBS buffer in an outer vial. The sealed vial was incubated in a shaker at 37 °C for a period of 3 days. At selected time intervals, 2 mL dialyzate was taken out from the vial and an equal volume of fresh PBS buffer was replenished to it. The released CPT was evaluated by measuring the UV-vis absorbance at 369 nm.

8.2.5 Characterization

The descriptions of ^1H NMR, FTIR, potentiometric titration, and TEM experiments have been presented in the previous chapters.

For the surface chemistry analysis, a Kratos Ultra X-ray photoelectron spectroscopy (XPS) system was used with a monochromatic Al Ka X-ray source operating at 15 kV and 10 mA. The corelevel spectra were obtained at a photoelectron take-off angle of 90°, measured with respect to the sample surface. Thermogravimetric analysis (TGA) was performed on a TA 2901 instrument under a nitrogen atmosphere (60 mL/min) at a heating rate of 10 °C /min from 40 to 600 °C. Dynamic light scattering (DLS) and zeta potential analyses were performed using a Malvern Zetasizer Nano instrument with a laser light wavelength of 633 nm, and all data were averaged over three measurements. For DLS of GO-CS, a bulk solution of 0.03 wt% was firstly filtered through a 0.8 μm filter at low pH (< 4) to remove dust and any other insoluble components, and the measurement angle was fixed at 173°.

For DLS of GO-CS/DNA, 100 μL of complexes containing 3 μg of pDNA were prepared at various N/P ratios. The mixture was vortexed for 6 s, incubated for 30 min and diluted to 1 mL by 15 mM of KCl solution at the time of measurement. The zeta potential measurements were carried out at 25 $^{\circ}\text{C}$ under the Smoluchowski approximation, and 20 to 100 runs were automatically chosen for a good reproducibility. The atomic force microscopic (AFM) measurements with the typical tapping-mode were performed using Digital Instrument S3000 AFM. The samples for AFM were prepared by dropping aqueous GO or GO-CS solutions (~ 0.01 mg/mL) onto a fresh silicon wafer, followed by drying it under ambient conditions for 24 h. To determine the CPT loading, UV-vis spectra of GO-CS and GO-CS/CPT were recorded by a Cary 50 Bio spectrometer. The CPT concentration was determined by its absorption at 369 nm (after subtracting the absorption contribution from GO-CS) using a molar extinction coefficient of $19900\text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. The amount of GO-CS was calculated from the absorbance at 800 nm.²³

8.2.6 Cell culture and cytotoxicity assay

Human hepatic and cervical carcinoma cells (HepG2 and HeLa) were obtained from ATCC (Rockville, MD). The cells were cultured according to the description in Chapter 7.2.3. MTT was used as an indicator of cell viability. In detail, 100 μL of cells were seeded into 96-well plates at a density of 1×10^5 cells/mL. After incubation for 24 h, the culture media were replaced with 100 μL of fresh media containing free CPT, GO-CS or GO-CS/CPT at different concentrations for further 20 h. After the cells were washed with phosphate-buffered saline (PBS) for three times, 10 μL MTT

(5 mg/mL in PBS) was added to the wells for 4 h. Then, the supernatant was removed by aspiration, and the formazan crystals were dissolved in DMSO (100 μ L/well), followed by shaking for 5 min. The absorbance was read using a microplate reader (Spectra Plus, TECAN) at a wavelength of 570 nm. For each sample, the final absorbance was the average of those measured from six wells in parallel.

8.2.7 *In vitro* gene transfection assay

The plasmid DNA used as a reporter gene was pRL-CMV (Promega, USA), which was amplified and purified via the method introduced in Section 7.2.4. The GO-CS's capability to inhibit pDNA migration was examined through gel electrophoresis experiments depicted in Section 7.2.4 as well.

For *in vitro* transfection studies using plasmid pRL-CMV, cells were seeded into a 24-well plate at a density of 5×10^4 cells per well in 0.5 mL of DMEM containing antibiotics for 24 h prior to transfection. At the time of transfection, the cells have grown to approximately 70–80% confluence, and the medium in each well was replaced with 0.3 mL of serum-rich medium. 20 μ L of complexes containing 0.5 μ g pDNA were added to each well and incubated with the cells for 4 h under a standard culture condition. Then the medium was replaced with 0.5 mL of fresh DMEM and cells were further incubated for 20 h under the same conditions, resulting in a total transfection time of 24 h. The luciferase assay was carried out according to the supplier's protocol (Promega, USA), and luciferase activity was measured on a single-well luminometer (Berthold lumat LB9507, Germany) for 10 s.

Scheme 8.1 Synthesis description of GO-CS

8.3 Results and discussion

8.3.1 Synthesis and characterization of GO-CS

As shown in Scheme 8.1, a carboxyl activating reagent (EDC) was utilized to initiate the formation of an amide linkage between GO and CS by forming an active intermediate, which was further stabilized by adding NHS.^{29,35} Relatively low molecular weight CS ($M_n = 3$ kDa) was chosen to avoid crosslinking. At the same time, an excess of CS was used to guarantee a more complete consumption of carboxylic groups on GO sheets, and the removal of unreacted polymer and impurities was achieved by filtration and dialysis. Figure 8.1 shows the ^1H NMR spectra of CS and GO-CS. The ^1H NMR spectrum of GO (not shown) shows there are no obvious resonance peaks between 0 and 4 ppm. For CS and GO-CS, the characteristic chemical shifts of CS near 2.0, 3.1, 3.5–4.0 ppm can be clearly distinguished. It is worth noting that the diamagnetic ring currents in graphene can induce significant upfield shift of the proton signals in CS's acetyl group and glucosamine backbone (H3–H6), which change from $\delta = 2.0$ and 3.5–4.0 to 1.9 and 3.4–3.9, respectively. Similar phenomena on polymer functionalized single-walled carbon nanotubes

(SWCNTs) were also observed and reported.³⁶⁻³⁷ FTIR spectral comparison between CS and GO-CS provides further information to confirm the successful polymer attachment to the surface of graphene sheets (shown in Figure 8.2), even though most of absorbance peaks are overlapped. The small absorbance peak of GO-CS at 1740 cm^{-1} still can be identified to represent the residual carbonyl moieties ($\text{C}=\text{O}$) on both the basal plane and periphery of GO.³⁸⁻³⁹ Moreover, the characteristic signal of secondary amide ($\text{N}-\text{H}$ bending) shifts from 1540 cm^{-1} to 1570 cm^{-1} , indicating the presence of newly formed amide bonds between GO and CS, and also the influence of graphene's aromatic carbon multiple bonds.³⁸ The XPS wide spectrum and C 1s spectrum of GO-CS are presented in Figure 8.3. Compared with the XPS scan spectrum of GO,³⁹ there is a new peak at ca. 397 eV corresponding to the N 1s binding energy from CS chains. From the C 1s XPS spectrum of GO-CS, the C 1s band can be fitted to several components, corresponding to carbon atoms in different positions: C-C (284.6 eV), C-N (286.0 eV), C-O (286.3 eV), C=O (287.8 eV), and O-C=O (289.2 eV), respectively.¹²

Figure 8.1 ^1H NMR spectra recorded in $\text{D}_2\text{O}/\text{DCl}$ for CS and GO-CS.

Figure 8.2 FTIR spectra of CS and GO-CS.

Figure 8.3 (a) XPS survey scan spectrum and (b) deconvoluted XPS C 1s spectra of GO-CS.

TGA is a complementary technique that can reveal the composition and changes in thermal stability of the GO-CS. Figure 8.4 indicates that GO itself is thermally unstable and starts to lose mass upon heating even below 100 °C, which is attributed to the volatilization of stored water in its π -stacked structure.⁴⁰ The major mass loss occurs close to 180 °C, which is attributed to the pyrolysis of labile oxygen-containing groups.^{3,41} In contrast, grafted CS chains seem to be effective in enhancing the thermal stability of GO sheets. TGA data for the GO-CS shows a 62% weight loss in a nitrogen atmosphere at 600 °C, whereas GO and pure CS have weight losses of 39% and 75%, respectively. Therefore, we can calculate that the composite contains about 36 wt% GO and 64 wt% CS. Serving as an alternative method, potentiometric titration also can help to estimate the composition by gradually neutralizing protonated amino groups along the CS branches.^{29,35} The experiment was conducted by titrating 25 mL of a 0.1 wt% composite solution (dissolved in 0.05 M HCl) with 0.1 M NaOH. As shown in Figure 8.5, the consumed molar amount of titrant NaOH between two inflection points for CS is 0.095 mmol. Considering that the amidation ratio of amino groups was so small that the DD (~90%) of CS could not have changed substantially, we can determine the weight percentage of GO in the composite is 32% and CS is 68%, which are very close to the results obtained from TGA.

Figure 8.4 TGA curves for (a) pristine GO, (b) GO-CS and (c) CS at a heating rate of 10 °C/min in nitrogen.

Figure 8.5 Potentiometric titration curve for 0.1 wt% GO-CS in an excess of 0.05 M HCl solution. The inset represents the difference plot of the pH curve.

Owing to the incorporation of the hydrophilic CS component, which has a pK_a of ~ 5.5 ,²⁸⁻²⁹ the polymer-grafted GO sheets can be readily dissolved in water with the aid of moderate sonication and also exhibit a typical pH-responsive association behavior. The colloidal size distributions of GO and GO-CS in aqueous solutions were characterized by the DLS technique. The intensity-average size value of GO (sonicated under the same conditions as the GO-CS's preparation process) in pure water located at about 170 nm with a PDI of 0.38 at 25 °C, which was close to the values from recent reports.^{8,42} After functionalized with CS, the average size of GO-CS evolved significantly as a function of the pH value (as depicted in Figure 8.6a). It is shown that the average size around 300 nm is firstly obtained below pH 4, but the size decreases to 210 nm near pH 6. Upon addition of NaOH, remarkable aggregation occurs upon increasing the pH to 11, as judged by the corresponding size increase from 210 nm to 1 μ m. Figure 8.6b presents the images showing the disperse status of GO-CS in various pH aqueous solutions with the same concentration after being stored for two weeks. It is observed that GO-CS can be uniformly dispersed in acidic water ($pH < 7$) to result in homogenous suspensions which are stable over a long time, but it fails to be well dispersed in the alkaline solution ($pH \sim 11$) and precipitates quickly after stopping sonication. This functionalization totally reversed the property of GO sheets which dissolve only in alkaline aqueous solutions but precipitate in acidic ones.^{12,43} In addition, the change in zeta potential according to pH variation was summarized in Figure 8.6a. The zeta potential of GO-CS sheets is greater than +40 mV at pH 2.9, and it decreases continuously to +6.7 mV when the pH is increased to

7, which reflects the ongoing deprotonation of the CS branches.²⁸⁻²⁹ Finally, the averaged zeta potential approaching 0 mV at pH > 8 means that the electrostatic charges are disappearing from the colloidal surface. On the basis of the experimental evidences from potentiometric titration, DLS, and zeta potential, the conformational changes of GO-CS at different pH values was schematically illustrated in Scheme 8.2. At pH < 4, the amino groups on CS chains are fully protonated and the GO-CS composites are individually dispersed in water due to strong electrostatic repulsion. The increased average size should be ascribed to the extended and semi-rigid CS branches on the GO sheets. At pH 5–7, a majority of CS segments are deprotonated and the intra- and inter-polymer repulsions decrease, resulting in the rolling up of the polymer chains. Meanwhile, the residual Coulombic forces are still stronger than the hydrogen-bonding between CS and GO domains, thus preventing the formation of large aggregates, which explains why a minimum size is achieved in this pH range. At pH > 8, entirely neutralized GO-CS sheets gradually aggregate and precipitate due to the hydrophobic association and strong hydrogen-bonding interactions.^{28,44} Additionally, GO and its derivatives may dissolve in water but aggregate in physiological solutions rich in salts or proteins due to the screening of electrostatic charges and nonspecific proteins binding, further debase their therapeutic effect.²⁰ The dispersion status of GO and GO-CS in PBS buffer and the cell culture medium were also investigated at the same concentration after 48 h (Figure 8.6c). It was found that GO-CS could be uniformly dispersed both in PBS buffer and the cell medium yielding homogenous and stable solutions while GO failed. Thus, it can be concluded

that the solubility of GO-CS was substantially enhanced due to the benign solubility of low molecular weight CS.

Figure 8.6 (a) Variations of particle size and zeta potential as a function of pH value for 0.03 wt% GO-CS. (b) Digital image for the long-term dispersion status of 0.1 wt% GO-CS in different pH valued solutions. (c) Digital image for the short-term dispersion status of 0.03 wt% GO (i & ii) and GO-CS (iii & iv) in PBS buffer and cell culture medium, respectively.

Scheme 8.2 Schematic description for the pH-responsive association behavior of the amphiphilic GO-CS nanocomposite

AFM provides a direct method to characterize the morphology and the thickness of GO and GO-CS sheets. As shown in Figure 8.7, the pristine GO sheets remain as micron-sized platelets, while the functionalized GO sheets have sizes in the range of hundreds of nanometers due to the sonication treatment during the synthesis procedure. The measured thickness of the GO sheets is uniform (~ 0.8 to 1.2 nm), which is consistent with those in the previous reports, suggesting the complete exfoliation of GO sheets down to individual ones.^{13,42-43} The topographic height of GO-CS increases to about $4\text{--}5$ nm, indicating that the CS chains were successfully grafted onto the surface of GO sheets. It is also worth noting that GO sheets exist with very sharp edges and flat surfaces. In contrast, the edges of GO-CS's appear relatively coarse and some protuberances can be observed on the surfaces, which are mainly generated from the polymer wrapping and folding. The similar phenomenon has been

widely unveiled in the recent studies on polymer immobilized graphene sheets.^{10,41-42,45-46} Of course, although GO and GO-CS were primarily exfoliated to monolayer platelets, a minor portion of the nanomaterial would still remain as stacked layers. Another possible contribution to this roughness may be attributed to the crumpling of graphene nanoplatelets.

Figure 8.7 Tapping mode AFM images for (a) GO and (b) GO-CS.

8.3.2 Drug loading and *in vitro* cell toxicity

Many anticancer drugs are aromatic and hydrophobic, resulting in poor water solubility, which generally hampers their clinical applications. Compared with their carbon nanotube (CNT) analogues, graphene derivatives are more attractive drug carrier because both sides of a single sheet are accessible for drug binding, and a

recent report also found that the 2D shape and ultra-small size of PEGylated graphene sheets showed interesting *in vivo* properties including highly efficient tumor passive targeting and relatively low retention in reticuloendothelial systems.²¹ Herein, we applied the GO-CS as a novel nanocarrier for CPT, a potent topoisomerase I inhibitor, in order to increase the solubility of CPT in aqueous systems for the first time. Furthermore, the cell toxicity tests for the GO-CS/CPT complex were carried out using HepG2 and HeLa cancer cells.

The loading of CPT was fulfilled by directly mixing CPT's DMSO solution with a GO-CS aqueous solution. Note that the excess, unbounded CPT precipitate was removed by centrifugation and filtration, and dialysis was used to remove any residual free CPT and DMSO. Enlightened by the recently reported NGO-PEG-SN38 complexe,²⁰ the binding of CPT onto GO-CS sheets was driven by hydrophobic interactions and π - π stacking between CPT and the intact graphitic regions of GO.²² Compared with the averaged colloidal size of GO-CS sheets (ca. 328 nm) determined by DLS, the size of the drug loaded GO-CS nanoplatelets increased to ca. 426 nm, suggesting a large amount of CPT adsorption. More convincing evidence came from UV-vis spectroscopy experiments carried out on GO-CS aqueous solutions with or without loading CPT (Figure 8.8a). According to the optical absorbance data and molar extinction coefficients of CPT and GO-CS, we can find that the loading capacity (about 20%, estimated from the weight ratio between drug and carrier) of GO-CS is twice more than that of those reported carriers, such as PEGylated-NGO or FA-NGO (folic acid conjugated nanoscale GO).^{20,23} Compared to small molecules

covalently bonded on other carbon nanomaterials,⁴⁷ the physical adsorption of drugs on GO-CS would maximally preserve the biological activity owed to the lack of chemical reactions between them.⁴⁸ The *in vitro* release profile of CPT from GO-CS in PBS buffer at 37 °C is shown in Figure 8.8b, and the cumulative plateau value of ca. 17.5% CPT release was reached upon 72h. Since CPT was tightly bound to the surface of graphene sheets via strong π - π stacking, other than being encapsulated in the polymeric micelles or nanoparticles,⁴⁹⁻⁵⁰ the GO-CS-CPT complex exhibited the outstanding stability and a high loading capacity.⁴⁸

Figure 8.8 (a) UV-vis absorption spectra of GO-CS and GO-CS-CPT with different concentrations. The inset represents the linear relationship between drug and carrier's concentration for the samples 1–5. (b) *In vitro* release profile of CPT from GO-CS-CPT in PBS buffer (pH 7.4) at 37 °C.

To investigate the enhancement of therapeutic efficacy by GO-CS, HepG2 and HeLa cells were incubated in a medium containing various concentrations of free CPT, GO-CS, and GO-CS-CPT. The relative cell viability was then measured by methylthiazoletetrazolium assay. As indicated in Figure 8.9a, there is no obvious toxicity (> 80% cell viability) measured for pure GO-CS, even at a high concentration of 100 mg/L for HepG2, revealing that the carrier alone is not cytotoxic and the enhanced efficacy should be attributed to the contribution of the drug bound to GO-CS. In comparison with free CPT, the water soluble GO-CS-CPT complexes induced a more significant cytotoxic effect on the tumor cells (Figure 8.9b). The GO-CS-CPT exhibits a potency with a 50% growth inhibition concentration (IC₅₀) of ~29 μ M, while free CPT displays a ~20% growth inhibition near the same concentration. In

other words, GO-CS can improve drug efficacy without increasing the chemotherapeutic drug dose. Since the CPT molecules go through the plasma membrane by passive diffusion, a high dose is needed to achieve it. Drug and GO-CS complexes may be internalized by cells mainly through an efficient endocytic process.^{20,23} The positive charges of CS may also improve the cellular uptake of GO-CS/CPT via electrostatic interaction with the negatively charged cell membrane. Moreover, one can speculate that the attached CPT can be protected from drug degradation by intracellular enzymes and affinity with serum albumin, therefore preserving its anticancer activity.⁵⁰⁻⁵² Similar cytotoxicity of GO-CS-CPT was also testified on HeLa cells (Figure 8.9c), and remarkable cell apoptosis can be observed at a low CPT dose.

Figure 8.9 *In vitro* cell toxicity assay. (a) Relative cell viability of HepG2 cells treated with GO-CS and GO-CS/CPT at various concentrations. (b) Relative cell viability of HepG2 cells treated with GO-CS/CPT and free CPT versus CPT concentration. (c) Relative cell viability of HeLa cells treated with GO-CS/CPT and free CPT versus CPT concentration.

8.3.3 GO-CS/DNA complexation and *in vitro* transfection

A successful gene delivery requires that pDNA should be condensed by cationic carriers into nanoparticles to facilitate cellular uptake.⁵³⁻⁵⁴ The condensation capacity of GO-CS was evaluated by agarose gel electrophoresis, DLS and zeta potential measurements, as well as TEM imaging. Positively charged carriers were mixed with negatively charged plasmid pRL-CMV to form the complexes at various nitrogen (N)/phosphate (P) (or N/P) ratios. Figure 8.10a shows the gel retardation result for the GO-CS/DNA complexes with increasing N/P ratio. The migration of pDNA was completely retarded by GO-CS at an N/P ratio of 4, indicating that this nanocomposite still maintains good compact ability as similar as pristine CS.^{25,55} It is also noteworthy that the particle size and surface charge of complexes are important factors to facilitate their diffusion, extravasation through vascular fenestration, and cellular uptake. GO-CS/DNA complexes display decreased particle sizes with increasing N/P ratio as shown in Figure 8.10b, and nanoparticles in the diameter range of 100–200 nm with a narrow polydispersity can be obtained after reaching the N/P ratio of 4. Representative TEM micrograph of GO-CS/DNA complexes at N/P ratio of 8 is presented in Figure 8.10c. Near spherical nanoparticles with a mean diameter around 150 nm can be discerned, which is in good agreement with that determined by DLS considering the hydration effect.³⁵ Zeta potential is an indicator of the surface charge of nanoparticles, and the presence of significant amount of positive charges after pDNA complexation is the major driven force to prevent aggregation due to the electrostatic repulsion. As indicated in Figure 8.10b, the zeta potential of GO-

CS/DNA rises dramatically when the N/P ratio increases from 2 to 4. Such trend is almost consistent with what is expected from gel retardation and DLS results.

Figure 8.10 (a) Agarose gel electrophoresis retardation of pDNA by GO-CS at various N/P ratios. (b) Variations of particle size and zeta potential for GO-CS/DNA complexes as a function of N/P ratio. (c) A representative TEM micrograph for GO-CS/DNA complexes at the N/P ratio of 8.

The *in vitro* transfection efficiency of GO-CS/DNA complexes was assessed using luciferase as a gene reporter in HeLa cells. Figure 8.11 shows the gene transfection efficiency mediated by GO-CS carrier in the presence of serum in comparison with that of CS at an N/P ratio from 1 to 10. Despite the cell-type dependency, both GO-CS and CS vectors exhibit similar capability in transfecting HeLa cells. Physicochemically, the small difference on the transfection efficiency between them may be attributed to their close particle sizes over most N/P ratio range (e.g., D_h values of GO-CS/DNA and CS/DNA are about 160 nm and 130 nm at N/P = 8, respectively). The optimal N/P ratio of gene transfection for them is around 4, being in good agreement with previous reports.⁵⁵⁻⁵⁶ Not surprisingly, the unmodified CS component was not quite efficient for HeLa cells transfection, as the relative luciferase activity obtained 24 h after transfection was below 10^5 .^{54,57-58} Under the

same conditions, PEI (25kDa) mediated transfection boosted the relative luciferase activity up to 2.6×10^5 at N/P = 10. The low level of transfection efficiency of CS/plasmid complexes on mammalian cells has been widely reported, but the transfection mechanism still remains controversial. According to Erbacher et al.'s investigation, CS/DNA complexes may not be endocytosed or may be sequestered in endocytotic vesicles, ending by DNA degradation in lysosomes.⁵⁶ It was also reported that CS conjugated DNA nanoparticles are most likely to condense into large aggregates and absorb to the cell surface. Thereafter, the complexes are internalized, and possibly released from endosomes due to the swelling of lysosomes along with the swelling of complexes, causing the endosome to rupture. Finally, these complexes were observed to accumulate in the nucleus.⁵⁹ Thus, Dastan et al. and Lee et al. concluded that the dissociation ability of the CS/DNA complexes is limited, which interferes with the transport of genes into nucleus or their transcription.^{57,60}

Figure 8.11 *In vitro* gene transfection efficiency of GO-CS/DNA complexes in comparison with that mediated by pristine CS at different N/P ratios in HeLa cells in presence of serum after 24 h. Naked pDNA (ND) and 25kDa PEI at its optimal N/P = 10 were used as positive controls. Data represent mean $\pm$ standard deviation ($n = 3$).

Even though the transfection efficiency of CS or our GO-CS is still not superior to that of commercial non-viral gene vectors, they possess some positive characteristics required for *in vitro* gene delivery, such as the ability to condense pDNA to form small and stable complexes, and low cytotoxicity. Most importantly, the incorporation of GO bestows the nanocarrier an excellent drug loading and release capability, which paves a new way for future studies focusing on the simultaneous delivery of anticancer drugs and therapy genes to enhance the therapeutic effectiveness.

8.4 Summary

We have reported for the first time the use of the chitosan-functionalized graphene oxide (named as CS-GO) as a nanocarrier to separately deliver a water-insoluble anticancer drug (CPT) and a DNA plasmid (pRL-CMV) into human cancer cell lines. Before the drug and gene delivery studies, the chemical structure, composition and morphology of the synthesized GO-CS nanocomposite were well characterized by ^1H NMR, FTIR, XPS, TGA, potentiometric titration, and AFM. The grafted CS branches endowed the resulting GO sheets with high solubility and stability in acidic and physiological aqueous solutions. More interestingly, GO-CS revealed a special pH-responsive association behavior as examined by DLS and zeta potential measurements. Due to the π - π stacking and hydrophobic interactions, GO-CS possesses a superior efficiency on binding CPT with a high loading content near 20 wt%. The *in vitro* drug release experiment showed that 17.5% CPT could be released after 72 h at 37 °C in PBS buffer. The pure nanocarrier was proved to be practically nontoxic to HepG2 and HeLa cells, and the GO-CS/CPT complexes were found to afford a strong potency to kill cancer cells *in vitro* as evaluated by MTT assay. Furthermore, as the nanocarrier is cationic, it was able to form electrostatic complexes (having particle sizes lower than 200 nm at optimal N/P ratios) with plasmid DNA, and a reasonable gene transfection efficiency was then achieved by the complexes in HeLa cells.

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Chapter 9

Conclusions and Recommendations for Future Studies

9.1 Conclusions

9.1.1 Interactions between ionic surfactants and polysaccharides

Molecular interactions between three polysaccharides (MC, CS, and KC) and two ionic surfactants (SDS and CTAB) have been studied respectively by isothermal titration calorimetry in Chapter 3. MC-SDS exhibited strong hydrophobic interaction as evidenced by the endothermic peak, followed by a weak ion-dipole interaction. KC-SDS did not show any ionic interaction as expected, but exhibited weaker hydrophobic interactions than MC-SDS. The strong ionic interaction was found for CS-SDS, followed by hydrophobic interaction between some hydrophobic moieties of CS and SDS tails. Titration of CTAB to MC resulted in a similar pattern of polymer-surfactant interaction to that for the titration of CTAB into CS. KC showed the strong interaction with CTAB by largely reducing the high endothermic enthalpy changes caused by the demicellization of CTAB. When three types of interactions are possible simultaneously, a general rule determines the order (or priority) for a surfactant to bind to a polymer according to the sequence ionic interaction (i.e., electrostatic interaction) > hydrophobic interaction > ion-dipole interaction. In addition, the ionic binding of an ionic surfactant to an ionic polymer is achieved through the surfactant ionic heads binding to the ionic groups of polymer chains, while the hydrophobic binding is completed through the surfactant tails that bind to the hydrophobic moieties

of polymer chains.

9.1.2 CTAB effects on CS-TPP nanoparticles

A systematic study of the influence of CTAB on the physicochemical properties and microstructures of CS-TPP nanoparticles has been carried out in Chapter 4. The particle size, zeta potential, dynamic behavior, morphology and electrostatic interactions between the nanoparticles and CTAB have been determined by means of DLS, SLS, zeta potential and TEM measurements. The addition of CTAB could increase the colloidal stability and reduce the hydrodynamic diameter of nanoparticles effectively in the salt solutions, particularly at high concentrations of CS. Under the experimental conditions, the CS-TPP nanoparticles experienced an overall increase in zeta potential but inconspicuous changes in particle size, by adding CTAB. A critical overlap concentration c^* of CS around 1.0 mg/mL was used to explain the abrupt changes in particle size. The combination of DLS and SLS methods was firstly applied to study the CS-TPP nanoparticles. It was found that the ratio $\rho (=R_g / R_h)$ decreased approximately from 0.9 to 0.5 with increasing CS concentration, and the salt and CTAB did not affect the ratio ρ significantly. Finally, the effect of CTAB concentration on the formation of CS-TPP nanoparticles was studied. In a semi-dilute solution of CS, the minimum particle size could be reached near the cmc of CTAB. The mechanism behind the phenomenon has been proposed and discussed.

9.1.3 Syntheses and characterizations of well-defined graft CS copolymers

The comb-structured CS-*g*-PDMAEMA was prepared using ATRP and active

ester conjugation and studied in Chapter 5. The successful application of ATRP bestowed PDMAEMA side chains of the CS backbones a narrow molecular weight distribution, and the intermediates were characterized by FTIR, ^1H NMR and GPC. The kinetic studies on the polymerization of DMAEMA initiated by NBPS revealed that the reaction was well controlled and a uniform PDMAEMA was obtained. The association behavior of the graft copolymer in aqueous solution changed significantly with varying the pH value of the medium, as investigated by LLS and TEM. At pH ~ 4 , the copolymer behaved as the Gaussian chains; at pH 5–6, the core-shell structured micelles were achieved via self-assembly; at pH ~ 7 , the core-shell structure further collapsed into double-layered hard spheres; and at pH ~ 8 , the micelles aggregated and precipitated. The potentiometric titrations and zeta potential tests were also carried out to understand and verify the factors leading to these interesting phenomena.

In Chapter 6, a novel graft terpolymer, CS(-g-PDMAEMA)-g-PNIPAM, capable of responding to two types of stimuli, was synthesized via the combination of ATRP and click chemistry. At first, PDMAEMA and PNIPAM were synthesized via ATRP, followed by substituting the halide end groups with azido groups. After converting CS into *alkynyl*-CS via amidation, PDMAEMA- N_3 and PNIPAM- N_3 side chains were successfully grafted onto the CS backbone via click chemistry, leading to the well-defined graft terpolymer. Thermo- and pH-responsive micellization behavior of the graft terpolymer in aqueous solutions was investigated by ^1H NMR, LLS, surface tensiometry, zeta potential and TEM. The core-shell structured micelles with

PNIPAM as a core and CS/PDMAEMA as a shell were formed in acidic environment ($\text{pH} < 4$) at elevated temperature ($> 38\text{ }^{\circ}\text{C}$), whereas the unimers formed polymeric micelles with CS/PDMAEMA cores in alkaline solutions ($\text{pH} > 7$) at room temperature. This work represents the first example of nonlinear dual hydrophilic CS copolymer showing a micellization behavior induced by two types of stimuli.

9.1.4 Thermo-responsive CS(-*g*-PDMAEMA)-*g*-PNIPAM terpolymeric carriers for *in vitro* gene delivery

Based on the synthesis route described in Chapter 6, a series of well-defined graft CS terpolymers (TCS) were developed as gene carriers and investigated in Chapter 7 as well. These TCS carriers demonstrate much lower cytotoxicity compared to the PDMAEMA and PEI controls, and good capability to complex plasmid DNA into nanoparticles for gene transfection. The optimized gene transfection efficiency achieved by TCS could be comparable to PEI (25kDa). At temperature above the LCST, tight and stable complexes are formed due to the collapse of PNIPAM chains in TCS. Reducing temperature below LCST facilitates the dissociation of TCS/DNA complexes and exposure of pDNA. We adopted two protocols to examine the thermal regulation of transfected gene expression: (i) the constant temperature approach (i.e. the culture temperature was kept at $37\text{ }^{\circ}\text{C}$); and (ii) the variable temperature approach (i.e. the transfected cells were incubated at $37\text{ }^{\circ}\text{C}$ for 18 h, $20\text{ }^{\circ}\text{C}$ for 2 h, and $37\text{ }^{\circ}\text{C}$ for 24 h. Thus, the level of gene expression can be thermally modulated after the internalization of TCS/DNA complexes in COS7 and HeLa cells. For the first time, we reported that the thermo-responsive gene transfection efficiency is also influenced

by the PNIPAM weight ratios in TCS carriers, and only TCS with NIPAM/DMAEMA molar ratio larger than 2 can show obvious thermally modulated gene transfection.

9.1.5 CS grafted graphene oxide as a nanocarrier for drug and gene delivery

The covalent functionalization of graphene oxide (GO) with CS was successfully accomplished via a facile amidation process. The CS grafted GO (GO-CS) sheets consisted of about 64 wt% CS, which imparted them with a good aqueous solubility and biocompatibility. Additionally, the physicochemical properties of GO-CS were well studied using various methods. As a novel nanocarrier, GO-CS was applied to load a water-insoluble, anticancer drug, namely camptothecin (CPT), via π - π stacking and hydrophobic interactions. It was demonstrated that GO-CS possessed a superior loading capacity for CPT, and the GO-CS-CPT complexes showed a remarkably high cytotoxicity in HepG2 and HeLa cell lines compared to the pure drug. At the same time, GO-CS is also able to condense plasmid DNA into nano-sized and stable complexes, and the resulting GO-CS/DNA nanoparticles exhibited reasonable transfection efficiency in HeLa cells at certain N/P ratios. Therefore, our GO-CS nanocarrier is able to load and deliver both an anticancer drug and a gene.

9.2 Recommendation for future studies

9.2.1 Well-defined graft CS copolymers for siRNA delivery

Considering the prominence of the functional polymers that have been grafted to CS to endow it versatile properties suitable for plasmid DNA delivery, selected siRNA can be employed to complex with those grafted CS copolymers to form

nanoparticles and their gene silencing efficiency can be studied. Compared to other antisense strategies such as antisense DNA oligonucleotides and ribozymes, RNAi is much more potent. Although the formation of copolymer/siRNA nanoparticles is a vital requirement, the stability and extracellular siRNA protection is equally important. After cellular uptake of the nanoparticles by endocytosis, endosome lysis and complex disassociation are needed to allow RNA-mediated gene silencing by forming RNA-induced silencing complex (RISC). The appropriate balance between protection and release plays a crucial role in the biological functionality of siRNA. It is also interesting to compare the DNA and siRNA transfection efficiencies using the graft CS copolymers which can be easily tailored by a “grafting onto” route. The extra vulnerability of RNA to enzymatic degradation may offer additional difficulties to CS derivatives-mediated RNA transfer, and make it more challenging than a conventional pDNA delivery. In addition, since the structure and size of siRNA are quite different from that of pDNA, the influence of different formulation parameters needs to be fully characterized with respect to the physical and biological features of the formed siRNA complex.

9.2.2 Co-delivery of anticancer drug and therapy gene by graphene-based nanocarriers

The cytotoxic anticancer drugs are the major agents for cancer chemotherapy although they have many limitations, such as poor water solubility, high toxicity to normal tissue and susceptibility to induce drug resistance. Gene therapy has been reported to offer a promise to overcome MDR phenotype, with cancer being treated

more effectively through the introduction of genes into cancer cells. Thus, combined anticancer drugs and gene therapies would be an effective strategy for cancer therapy. Graphene and its derivatives have been found to be good candidates for anticancer drugs' loading and delivery. By introducing various cationic polymers (e.g. PEI and PDMAEMA) onto graphene-based nanomaterials, simultaneous delivery of a drug as an apoptosis inducer and siRNAs as suppressors of pump resistance and cellular antiapoptotic defense will be achieved to enhance the therapeutic effects.

-THE END-