Journal of Applied Polymer Science

REVIEW

Poly(L-histidine)-Containing Polymer Bioconjugate Hybrid Materials as Stimuli-Responsive Theranostic Systems

Renjith P. Johnson, Johnson V. John, Il Kim

BK 21 PLUS Center for Advanced Chemical Technology, Department of Polymer Science and Engineering, Pusan National University, Pusan, Republic of Korea

Correspondence to: I. Kim (E-mail: ilkim@pusan.ac.kr)

ABSTRACT: For decades, researchers have aspired to develop materials for noninvasive treatment and monitoring of pathological conditions. Various organs, tissues, subcellular compartments, and their pathophysiological states can be characterized by their pH values. pH-dependent intracellular tumor targeting has received particular attention due to the unique acidic environment of the solid tumors created by physiological and metabolical abnormalities. Responsive nanocarriers, when exposed to these pH stimuli, respond quickly to the physicochemical changes by undergoing structural deformations, such as swelling and phase transition, which favors the drug release specifically at the diseased site. Recently, researchers have developed several new poly(L-histidine) (p(His))-based pH responsive systems for sustained drug release and molecular targeting. This review focuses on the p(His)-based pH responsive nanocarriers, which are utilized in biomedical applications such as anti-cancer drug delivery and nucleic acid delivery. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40796.

KEYWORDS: biocompatibility; biomedical applications; drug delivery systems; proteins; ring-opening polymerization

Received 18 March 2014; accepted 31 March 2014

DOI: 10.1002/app.40796

INTRODUCTION

Nanovehicles made from biocompatible synthetic polymers are of increasing interest in the field of drug delivery as therapeutic agents. Polymers show superior pharmacokinetics than small molecule drugs, allowing longer circulation time and offering the opportunity for tissue targeting. Nanoparticles (20–300 nm size) initially accumulate passively in the diseased tissues by enhanced permeation and retention (EPR) effects. The origin of the EPR concept dates back to the late 1970s, when Maeda et al. [1,2] discovered the selective accumulation of macromolecular drugs in tumor tissues. The specific passive accumulation of macromolecules was attributed to the defective tumor vasculature with disorganized endothelium at the tumor site and to a poor lymphatic drainage system. Since then, researchers have exploited this process for the delivery of various drugs by conjugating the drug with polymers or encapsulating them within nanoparticles. The recent development of nanocarrier systems with engineered triggering mechanisms for drug release has offered a versatile strategy for cancer treatment and has attracted much attention. Importantly, stimuli-responsive polymers respond to a small or modest change in the microenvironment, such as changes in temperature, pH, light, or salt concentration, with a sharp change in their properties. This sensitive responsiveness can be exploited for the preparation of 'smart' delivery systems, which mimic biological response behavior to some extent.

The traditional nanosystems relied on the passive diffusion of drugs into the diseased sites.[1,3] New generation of antitumor therapeutics use triggered release from an intelligent carrier to deliver a high-dose of a desired drug at specific sites.[4–7] Undoubtedly, poly(L-histidine) ((p(His) is a desirable material for the construction of pH-sensitive polymeric carriers. The imidazole ring of p(His) (pK_b ~6.5) has lone pairs of electrons on the unsaturated nitrogen that endow it with pH-dependent amphoteric properties. The ionization of p(His) (below pK_b) switches the nature of this material from lipophilic to lipophobic.[6,8] Additionally, the p(His) exhibits a strong endosomolytic property due to its proton sponge effect and/or its interaction with anionic phospholipids in the endosomal compartments.[6,9,10] These properties of p(His) contributed to the development of a smart micellar delivery system responsive to tumor extracellular pH (pH_e).

Utilization of nanoparticles made of self-assembled polymers containing ionizable p(His) blocks that undergo pH_e-induced structural changes is an effective approach for achieving the triggered release to deliver a high-dose of drugs at their sites of action. The pH-sensitive polymeric carriers have been

Renjith P. Johnson is a Postdoctoral Research Fellow in the Department of Polymer Science and Engineering at Pusan National University. He completed his B.Sc. degree in Chemistry from Kannur University (2004) and received his M.Sc. degree in Chemistry from Bharathidasan University (2006), India. Recently, he received his Ph.D in polymer science and engineering under the supervision of Prof. Il Kim. His research focused on the development of novel polymer/lipid-bioconjugate hybrid materials for various biomedical applications.

Johnson V. John In 2008, he received Master's degree in Biochemistry from Bharathidasan University, India. During 2010-2011, he was a scientific assistant under the guidance of Dr. V. V. Pillay in department of forensic toxicology, Amrita Institute of Medical Science, kerala, India. Since 2011, he has been working towards his Ph.D under the supervision of Prof. Il Kim. His research focuses on developing thermoplastic elastomers and polymer-conjugated hybrid materials for biomedical applications.

Prof. Il Kim received his Ph.D. from Korea Advanced Institute of Science and Technology, Republic of Korea, in 1990, followed by postdoctoral training under Prof. Richard F. Jordan, Department of Chemistry, University of Chicago (1994–1995). Since 2001, he is a full chair at the Department of Polymer Science and Engineering, Pusan National University, Republic of Korea. His research interests include organometallic complex-catalyzed polymerizations of olefins and dienes, synthetic polymer bioconjugate hybrid materials and their biomedical applications, catalysts for the utilization of carbon dioxide as a monomer for its fixation in polymeric materials, and a variety of controlled polymerizations. He is also managing a BK21 PLUS Center for Advanced Chemical Technology, supported by the Ministry of Education, at Pusan National University. In addition, he is a deputy board member of the Korea Polymer Society and an Adjunct Professor at Cornell University, USA. He has supervised fifteen Ph.D. students and has authored more than 300 peer-reviewed scientific publications. He has received many awards, including the Yeosan Catalyst Award (Korean Institute for Chemical Engineers), the Science and Technology Award of Pusan City, Yeonam Fellow (LG Corp.), the PNU Premier Professor of the Year, and the Best Cooperation Researcher of the Year 2012 (Korea Polymer Society).

extensively used in targeted antitumor drug delivery.[11–15] These carriers relied on the intrinsic differences between the relative acidities of the tumors (pH 6.5–7.2) and the surrounding normal tissues for targeted delivery.[16,17] Development of thermo-responsive as well as pH/temperature dual-responsive systems have also been reported.[18]

The recent development of p(His)-containing pH-responsive polymers has allowed a new mode of antitumor drug delivery. Drug-carriers based on pH-sensitive polymers have been investigated for specifically targeting the tumor extracellular acidity[6,19] and the pH in endosomes. Two major approaches have been used to trigger drug release in response to a decrease in pH: self-assembled nanoparticles composed of polymeric blocks or components containing ionizable groups that undergo pH-dependent structural changes or destabilization.[20] Upon reaching their target sites, these nanocarriers undergo changes that alter their nature to induce aggressive interactions with tumor cells,[6,21] rapidly destabilizing and localizing at their intracellular destination,[22] and enhancing the drug release rate.[6]

Several published reports have described the synthesis of p(His)-containing block copolymers for anti-cancer drug delivery. The use of these pH-sensitive nanovehicles led to enhanced drug release and increased anticancer activity of the drugs. In this review, we discuss representative examples of the recent developments in triggered drug release nanosystems fabricated using stimuli-sensitive polymers containing p(His).

POLY(HISTIDINE) CONJUGATED POLYMER HYBRID MATERIALS FOR pH RESPONSIVE CELLULAR DELIVERY

Biodegradable polymers, both natural and synthetic, have been extensively studied as potential carrier materials for site-specific drug delivery because of their non-toxic, biocompatible nature. These biocompatible polymers have been used to fabricate nanoparticles of various shapes, sizes (micro or nano), biodegradability, and types of surface groups. Such particles have been routinely used as vehicles for the delivery of therapeutic agents due to their ability to protect the encapsulated drug, their ease of fabrication, tunable degradation kinetics, and high

surface to volume ratio, which allows the display of a large number of targeting agents and/or imaging probes.[23,24] Both natural and synthetic polymers modified with p(His) are promising novel biomaterials for the controlled delivery of drug and nucleic acids. In the following section, we provide an overview of the different p(His)-modified natural and synthetic polymers and their applications, focusing on controlled delivery of drugs, and nucleic acids.

NATURAL POLYMER-POLY(HISTIDINE) CONJUGATES FOR INTRACELLULAR DRUG AND GENE DELIVERY

Polymeric materials used for preparing nanoparticles for applications in drug or gene delivery must be biocompatible and preferably biodegradable. To achieve biocompatibility and biodegradability, many polymeric materials, including poly(lactica-cid), poly(glycolic acid), polycaprolactone, polysaccharides (particularly chitosan), poly(acrylic acid) family, proteins, and polypeptides have been used. Polysaccharides have attracted considerable attention as a popular material for preparing nanoparticles for intracellular delivery. The natural sources of polysaccharides include algae (e.g., alginate), plants (e.g., pectin), microbes (e.g., dextran), and animals (e.g., chitosan).[25] Polysaccharides have a large number of reactive groups, a wide range of molecular weights, and varying chemical composition, which contribute to their structural and functional diversity. Depending on the charge, polysaccharides can be divided into positively charged (chitosan) and negatively charged polysaccharides (heparin, hyaluronic acid).

Because of the presence of various derivatizable groups on molecular chains, polysaccharides can be readily modified chemically and biochemically to produce an array of polysaccharide derivatives. As natural biomaterials, polysaccharides are highly stable, safe, non-toxic, hydrophilic, and biodegradable. Additionally, their natural sources are abundant, and cost of processing is low. Most of the natural polysaccharides have hydrophilic groups, such as hydroxyl, carboxyl, and amino groups, which form non-covalent bonds with their partners in tissues (mainly epithelial and mucosal membranes).[26] These advantages allow their use in various biomedical applications. In recent years, polysaccharides conjugated with histidine derivatives or p(His) have been investigated for their potential application as nanoparticle drug and gene delivery systems.

Cho et al. reported the fabrication of *N*-acetyl histidine-glycol chitosan conjugates for the intracytoplasmic delivery of the anticancer agent paclitaxel (PTX).[27] These authors found that nanoparticles of self-assembled histidine-conjugated glycol chitosan (NAcHis-GC) is a promising system for the intracytoplasmic delivery of PTX. The *N*-acetyl histidine (NAcHis) was hydrophobic at neutral pH and the conjugates formed self-assembled nanoparticles with a mean diameter of 150−250 nm. These nanoparticles disassembled in slightly acidic environments, such as those in endosomes, due to a breakdown of the hydrophilic/hydrophobic balance initiated by the protonation of the imidazole group of NAcHis. Cellular internalization of the pH-sensitive self-assembled nanoparticles and the ensuing drug release were analyzed by flow cytometry and confocal microscopy. It was found that, NAcHis-GC nanoparticles internalized by adsorptive endocytosis were exocytosed or localized in endosomes. Further, the NAcHis-GC nanoparticles released drugs into the cytosol. Cell cycle analysis demonstrated that PTX-incorporated NAcHis-GC nanoparticles were effective in inducing growth arrest.

Chen et al. [28] prepared poly(His)-chitosan/alginate complex microcapsules from poly(His) in the presence of chitosan in acetate buffer at pH 4.6. The microcapsules obtained were spherical and well-dispersed with a smooth surface and narrow size distribution, and efficiently encapsulated hemoglobin. The authors reported that, depending on the molecular weight of chitosan, high encapsulation efficiencies (91.3%) were realized. Drug release from the microcapsules followed first-order release kinetics. Approximately 84.8%, 71.4%, and 87.3% of the encapsulated drug was released from microcapsules prepared using low, medium, and high molecular weight chitosan, respectively, during the 72-h incubation in phosphate buffer saline (PBS) at pH 6.8. Bae et al.[29] reported the development of self-organized nanogels capable of responding to tumor extracellular pH (MCF-7). The pH_e-responsive nanogel was developed by exploiting histidine chemistry using pullulan deoxycholic acid N_α-Boc-L-histidine. The doxorubicin (Dox)-loaded self-organized nanogels composed of hydrophobic pullulan-N_α-Boc-L-histidine, its response to pH_e, and their efficacy against MCF-7 cells were investigated.

Huang et al. reported the synthesis and characterization of nanoparticles based on histidine–hyaluronic acid conjugates as Dox carriers.[30] The histidine–hyaluronic acid conjugates were synthesized using hyaluronic acid as the hydrophilic segment and histidine as the hydrophobic segment by 1-ethyl-3(3-dimethylaminopropyl)carbodiimide-mediated coupling reactions. The structural properties of the histidine–hyaluronic conjugates were investigated using ^{1}H NMR spectroscopy. The characteristics of the histidine–hyaluronic nanoparticles were investigated using dynamic light scattering (DLS), transmission electron microscopy (TEM), scanning electron microscopy (SEM), and fluorescence spectroscopy. It was found that the particle size varied from 350 nm to 740 nm, depending on the degree of substitution of histidine. Fluorescence microscopy images indicated that the nanoparticles were taken up by the MCF-7 cell line, and which was significantly decreased by the competition inhibition of free hyaluronic acid. Cytotoxicity tests showed that Dox-loaded nanoparticles exhibited similar dose- and time-dependent cytotoxicity against MCF-7 as free Dox. These results indicate that self-assembled nanoparticles can be used in cancer therapy as carriers of hydrophobic drugs.

Recently, Chen et al. investigated hyaluronic acid-*g*-poly(L-histidine) conjugate as a carrier of anti-cancer drugs.[31] The hydrophobic p(His) was conjugated with hyaluronic acid to fabricate a pH-responsive and tumor-targeting copolymer. These authors studied the effect of the degree of substitution on the pH-responsiveness of the copolymer micelles. *In vitro* drug release studies demonstrated the Dox was released from the

micelles in a pH-dependent manner. *In vitro* cytotoxicity assays showed that the blank micelles were nontoxic. Results of the MTT assays suggested that Dox-loaded micelles with a low p(His) substitution were highly cytotoxic to MCF-7 cells overexpressing CD44 receptors. Cellular uptake studies revealed that these pH-responsive Dox-loaded polymer micelles entered the cells via receptor-mediated endocytosis. The results demonstrated that this copolymer could be used to construct biocompatible tumor-targeted and pH-responsive delivery vehicles.

Hwang et al. [32] developed a Dextran-*b*-p(His) copolymer nanoparticle for pH-responsive drug delivery into tumor cells. The copolymer was synthesized by attaching the reductive end of dextran to the amino groups of poly(His). These researchers fabricated pH-responsive nanoparticles incorporating Dox by combining the nanoprecipitation and dialysis methods, and characterized their properties as drug delivery vehicles using HuCC-T1 cholangiocarcinoma cells. These nanoparticles exhibited pH-dependent changes in particle size. The size of the nanoparticles increased at acidic pH and decreased at basic pH. The rate of Dox release from these particles was higher at acidic pH than at basic pH, demonstrating that the nanoparticles had pH-sensitive properties that allowed pH-dependent drug delivery.

Nonviral vector-mediated gene therapy is currently one of the most attractive alternative therapeutic strategies used and is free from the risks inherent in viral systems. However, the obstacles that limit their use include introduction of plasmid DNA (pDNA) into multiple cell types and efficient gene expression with limited toxic side effects. Dextrans are efficient nonviral delivery systems and exhibit several attractive properties, including superior biocompatibility, ease of use, biodegradability, availability, improved transfection efficiency, and minimal toxicity. Sharma et al. developed a dextran-histidine polycation system for nonviral gene delivery.[33] These researchers synthesized a histidylated dextran derivative modified with histidine as an endosomolytic agent, and characterized its physicochemical properties. Experiments with fluorescence tags were showed the route of internalization and intracellular trafficking of the polymer/pDNA complex in C6 cells.

SYNTHETIC POLYMER-POLY(HISTIDINE) CONJUGATES FOR INTRACELLULAR DRUG AND GENE DELIVERY

A number of pH-sensitive drug-delivery systems based on a combination of various synthetic polymers and p(His) that undergo structural destabilization at slightly acidic pH or in response to other stimuli have been developed. Bae's research group investigated polymeric nanovehicles composed of p(His) with engineered trigger release mechanisms for the delivery of various anticancer drugs.[34] For instance, a pH_e-sensitive multifunctional micelles with a ligand that binds its receptors only when exposed to an acidic tumor extracellular environment ($6.5 < pH < 7.4$) was developed. The ligand biotin was anchored to the surface of the micelles made from a combination of p(His)-polyethylene glycol (PEG) and poly(L-lactic acid) (PLLA) PLLA-PEG-p(His)-biotin. At neutral pH, the p(His) was insoluble and collapsed on the hydrophobic core surface. Thus, the biotin was buried in the PEG corona and was not available for binding. Once the micelles were exposed to the acidic extracellular space of tumors, the p(His) became soluble and exposed biotin for binding to the tumor receptors.[6] Although active targeting has benefits, such as enhanced efficacy, this strategy may lead to the accumulation of high levels of the polymeric micelles. Ineffective drug release lowered the antitumor efficacy of drugs. Currently, micelle-based anticancer therapy involves multifunctional micelles containing at least two out of three features regarding targeting ligand and extracellular or intracellular triggered drug release[7,35] (Figure 1). Combined use of these approaches will further improve the specificity and efficacy of micelle-based drug delivery. Among the multifunctional polymeric micelles, those are capable of active

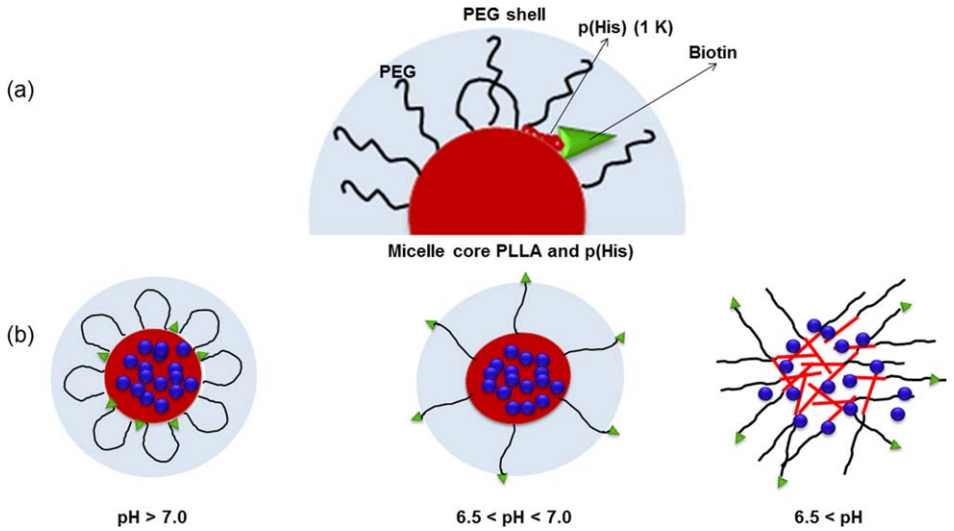

Figure 1. (a) Above pH 7.0, biotin anchored on the micelle core via pH-sensitive p(His) is shielded by the PEG shell of the micelle. (b) In acidic conditions ($6.5 < pH < 7.0$), biotin is exposed on the surface and interacts with cells, facilitating biotin-receptor-mediated endocytosis. When pH is further lowered ($pH < 6.5$), the micelle destabilizes, resulting in enhanced drug release and disrupting the endosomal membrane (Redrawn from *Nano Lett.* **2005**, 5, 325; Ref. 7). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

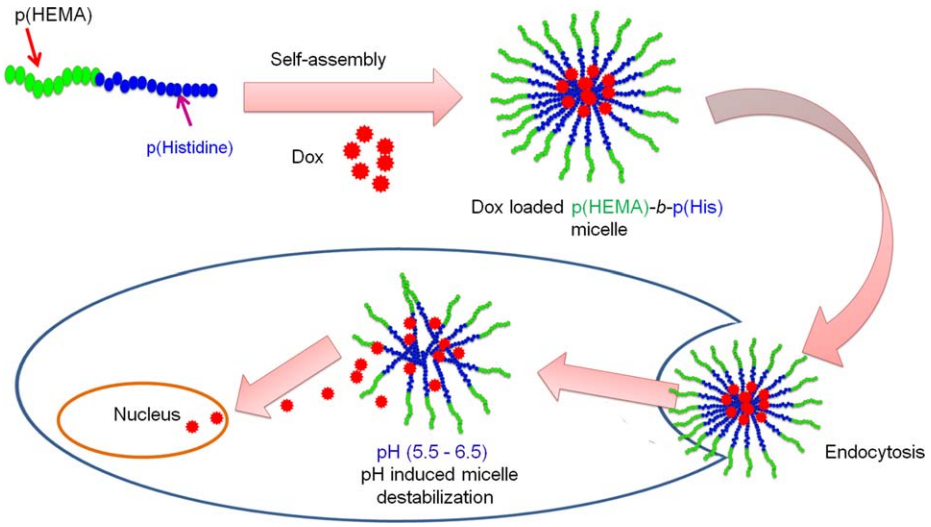

Figure 2. Diagrammatic representation of pH-responsive triggered drug release of Dox from p(HEMA)-*b*-p(His) micelles at tumor extracellular pH[20]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

targeting and pH-triggered release offers opportunities for improving therapeutic efficacy. Although excellent review articles on triggered drug release from pH-sensitive polymeric micelles have been published,[34,36] it is important to describe the recently developed p(His)-containing antitumor drug delivery systems.

Bae et al. [37] reported the development of a L-histidine-based pH-sensitive anticancer drug carrier micelle and a brief evaluation of its systemic toxicity. The Dox-carrier micellar system consisting of p(His)-*b*-PEG and PLLA-*b*-PEG-folate was developed for targeting the early endosomal pH. Studies demonstrated that intracellular high-dose strategy using such micelles is effective in overcoming the multidrug resistance (MDR) of cancer cells. To overcome the low Dox concentrations in the micelle solution and the lack of long-term stability of the micelles, stable and lyophilized micelle formulations were developed using excipients of sucrose, PEG, or Pluronic. The reconstituted micelle solutions were examined for particle size, pH sensitivity, and cytotoxicity, and the results were compared with that of the non-lyophilized micelles. Further, these authors evaluated the pH-sensitive mixed micellar system for MDR ovarian tumor targeting and optical imaging.[38] Dox-encapsulated pH-sensitive mixed micelles underwent folate receptor-mediated endocytosis. The drug uptake, endosomal disruption, and cell viability were investigated. MDR ovarian A2780/Dox^R xenografted nude mice models were used for examining *in vivo* tumor growth inhibition. The authors found superior efficacy of the folic acid conjugate to free Dox and a Dox loaded pH-insensitive micelle. Further, optical imaging using a Cy5.5 fluorescence dye-labeled mixed micelle system demonstrated that the novel micellar system can be used in diagnostics. For double-targeting folate receptor at early endosomal

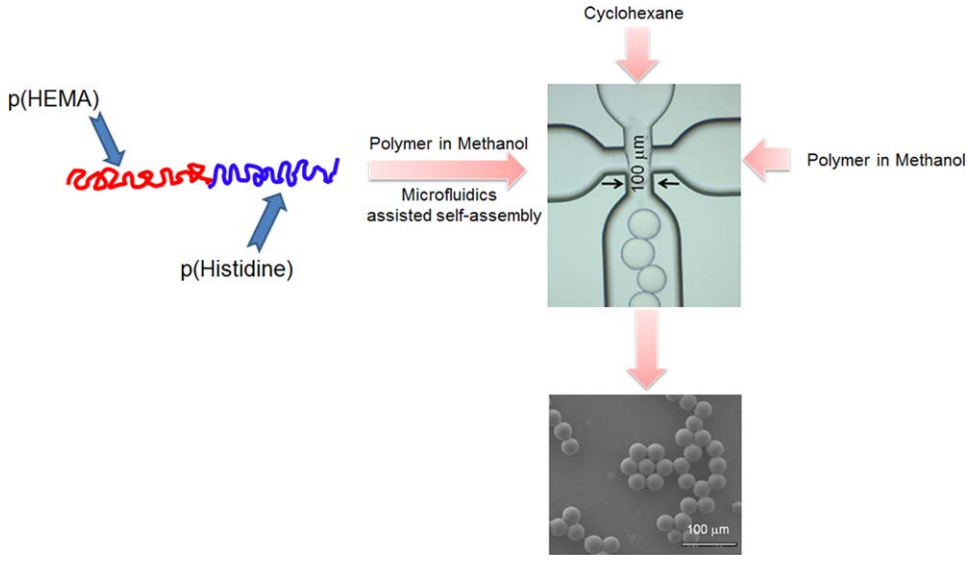

Figure 3. Schematic representation of the microfluidics-assisted fabrication of pH-responsive microspheres from p(HEMA)-*b*-p(His) block copolymer[41]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

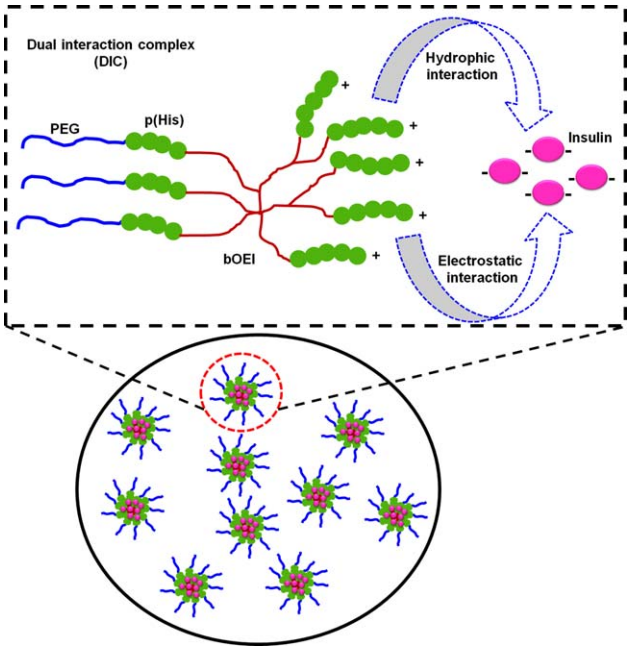

Figure 4. Diagrammatic representation of the dual-interaction complex (DIC)-loaded PLGA microspheres for insulin delivery (Redrawn from *Biomaterials* **2012**, 33, 8848; Ref. 43). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pH, a mixed-micelle system composed of p(His)-*co*-phenylalanine-*b*-PEG and PLLA-*b*-PEG-folate was developed.[39] Dox-loaded micelles effectively killed both wild-type (A2780) and Dox-resistant ovarian MDR cancer cells through an instantaneous release of high dose of Dox in the cytosol that was facilitated by active internalization and accelerated Dox release triggered by endosomal pH. Therapeutic efficacy of this micellar system [Dox-loaded micelles (Dox loading content: 20 wt %)] was also evaluated *in vivo* in MDR ovarian tumor-xenografted mouse models.[40]

Recently we designed and synthesized a biocompatible synthetic polymer bioconjugate hybrid material consisting of poly(2-hydroxyethyl methacrylate) (p(HEMA)) and (p(His)). The hybrid block copolymer was synthesized by combining atom transfer radical polymerization (ATRP) of HEMA with the ring opening polymerization (ROP) of benzyl-*N*-carboxy-L-histidine anhydride (His-NCA).[20] The resulting biocompatible and membranolytic $p(HEMA)_{25}$-*b*-$p(His)_n$ ($n = 15$, 25, 35, and 45) polymers were investigated for their use as pH-sensitive drug carriers for tumor targeting. Dox was encapsulated in nanosized micelles fabricated by a self-assembly process and delivered under different pH conditions. Micelle size was determined using DLS and TEM. The release of Dox from the micelles was sensitive to pH. Antitumor activity of the released Dox was assessed using the HCT 116 human colon carcinoma cell line. Dox released from the p(HEMA)-*b*-p(His) micelles was biologically active and dose-dependently killed cancer cells at acidic pH. The p(HEMA)-*b*-p(His) hybrid materials were capable of self-assembling into nanomicelles and effectively encapsulating the chemotherapeutic agent Dox, which allow their as tumor-targeted drug carriers (Figure 2).

As an extension of the above study, we fabricated highly uniform microspheres by microfluidics-assisted self-assembly of $p(HEMA)_{25}$-*b*-$p(His)_n$, block copolymer and utilized these microspheres as pH-sensitive carriers of the chemotherapeutic agent Dox and the beta-lactam antibiotic ampicillin.[41] Monodisperse microspheres of approximately 30 μm diameter and excellent structural integrity could be successfully fabricated using this method (Figure 3). The capacity of p(HEMA)-*b*-p(His) hybrid materials to self-assemble in microenvironments and to effectively encapsulate drug molecules allow their use as drug delivery systems.

To overcome the limitations of monomeric pH probes for acidic tumor environments, Bae et al. designed a mixed micelle pH probe composed of PEG-*b*-p(His) and PEG-*b*-PLLA.[42] The acidic pH-induced transformation of the mixed micelles showed a pH sensitivity within 0.2−0.3 pH units. It was found that the micellar pH probes sensed pH differences in PBS, cell culture medium, and rat whole blood. This micellar pH probe loaded with Dox may find applications in future therapy and diagnosis.

Na et al. developed a multi-arm histidine copolymer for controlled release of insulin from poly(lactide-co-glycolide) microspheres.[43] The star-shaped block copolymer made of methoxy poly(ethylene glycol) (mPEG), branched oligoethylenimine (bOEI), and p(His) was synthesized via the multi-initiation and ring-opening polymerization of His-NCA on bOEI with a PEG conjugation (Figure 4). The authors found that mPEG-bOEI-p(His) and insulin form a dual ionic and hydrophobic interaction complex at pH 7.4. When tested at various NaCl concentrations, this complex showed significant resistance to high salt-induced dissociation. When incorporated into the microspheres, the dual interaction complex was found to be evenly distributed. Unlike insulin-loaded microspheres, the complex-incorporated microspheres showed improved insulin release profile with a smaller initial burst and nearly zero-order release kinetics. Results of animal studies suggested potential application of this system for sustained protein delivery.

Recently we reported the use of p(His)-tagged 5-aminolevulinic acid (ALA) prodrugs as new photosensitizing precursors of protoporphyrin IX for applications in photodynamic therapy (PDT) of cancer.[44] A series of pH-sensitive ALA-$p(His)_n$ ($n = 5$, 10, 15) prodrugs were synthesized via ROP of His NCA initiated by the amine hydrochloride group of ALA. As an alternative to ALA in PDT, the synthesized prodrugs were used to treat the cultured human colon cancer cell line HCT116 under different pH conditions. The effect of ALA-$p(His)_n$ derivatives was evaluated by monitoring the fluorescence intensity of protoporphyrin IX and measuring the cell survival rate after irradiation (Figure 5). The assessment of cytotoxicity of ALA and ALA-$p(His)_n$ derivatives in HEK293T and HCT116 cells in the absence of light at pH 7.4 and 6.8 showed that the cell viability was relatively higher than 100%. ALA-$p(His)_n$ showed higher phototoxicity and selectivity than ALA under different pH conditions. Because the length of the histidine chain increases in the ALA-$p(His)_n$ prodrugs, the PDT effect was found to be superior.

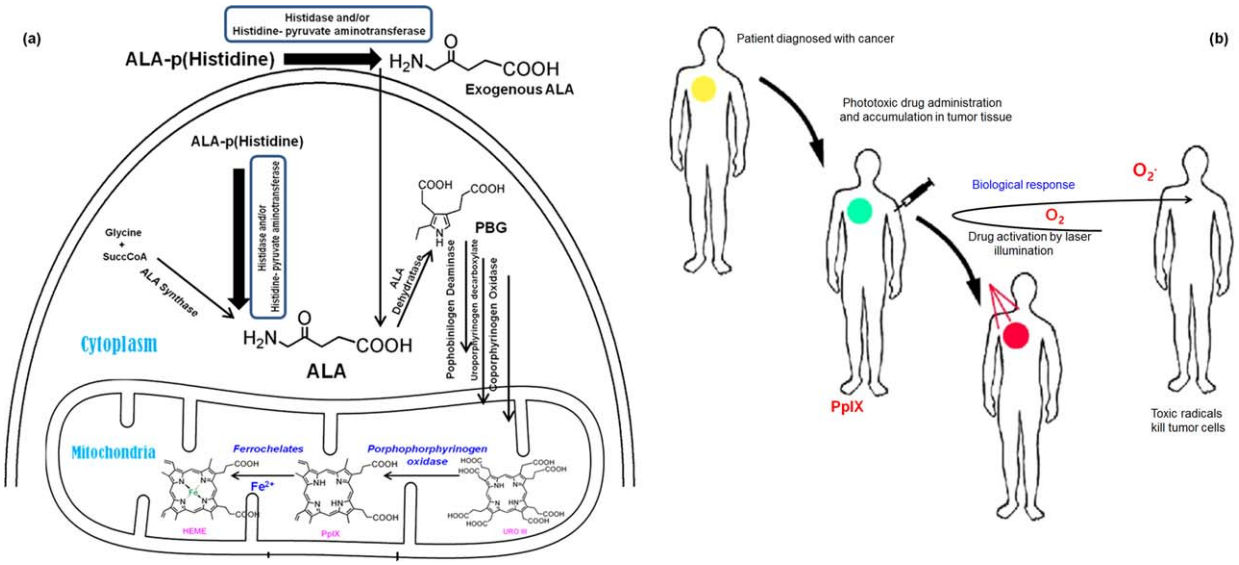

Figure 5. (a) Schematic representation of the cellular processing of ALA-p(L-His) and (b) various steps in a typical photodynamic treatment of cancer[44]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

A pH-responsive and charge-shielded cationic micelle composed of p(His)-*b*-PEI (short branched polyethyleneimine) was developed by Bae et al. [45] for use in acidic cancer treatment. The authors addressed cancer cell heterogeneity while endowing tumor specificity, and investigated the charge shielding/deshielding in vitro and *in vivo* with a PTX loaded cationic micelle prepared from a block copolymer of p(His) (3.7 kDa) and PEI (1.8 kDa). The surface of the cationic micelle was shielded by electrostatically complexing with a negatively charged mPEG (2 kDa)-*b*-polysulfadimethoxine (4 kDa) at pH 7.4. It was found that, the unshielded micelle at pH 7.4 and deshielded micelle at tumor extracellular pH were readily taken up by MCF 7 breast adenocarcinoma and SKOV-3 ovarian carcinoma cells, whereas the uptake of the shielded micelle at pH 7.4 was found to be minimal. These results suggested that this system have the potential to serve as a cationic nanoparticle for applications in

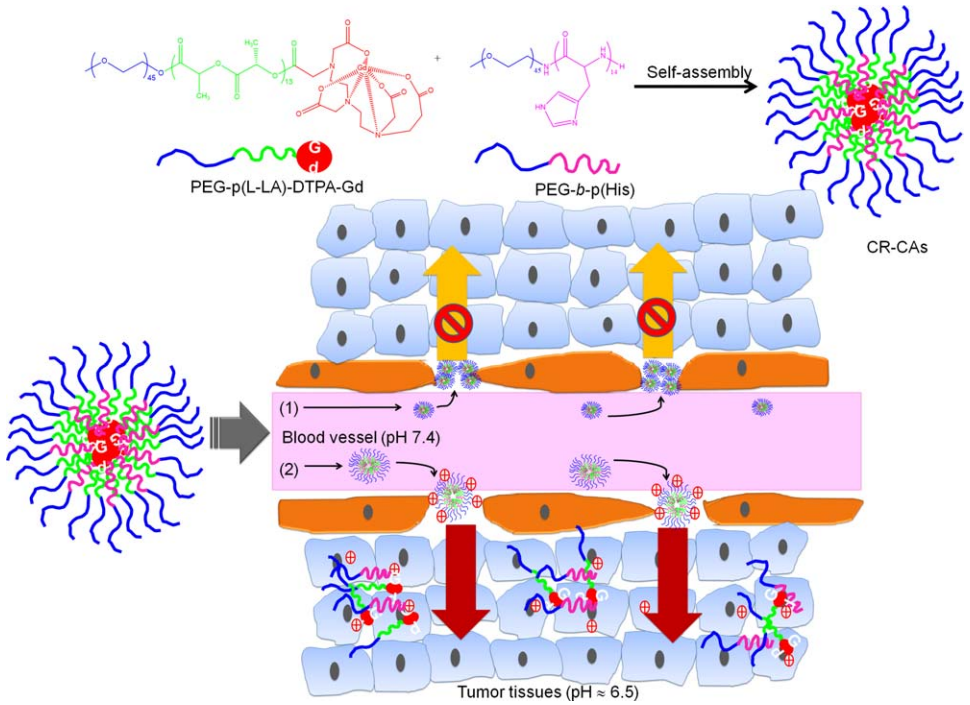

Figure 6. Diagrammatic representation of the preparation and self-assembly of the cancer-recognizing MRI contrast agents, the self-assembly into micelles in aqueous solution at pH 7.4, and the tumor-accumulation behavior (Redrawn from *Biomaterials* **2014**, 35, 337; Ref. 46). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

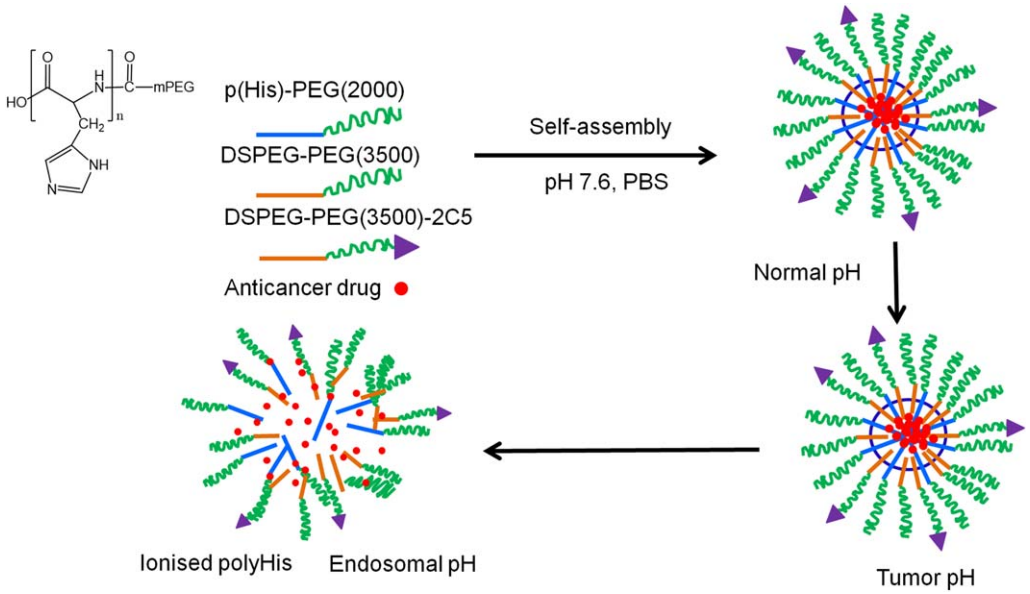

Figure 7. Acid-triggered drug release from the pH-sensitive mixed micelles (Redrawn from *Biomaterials* **2013**, 34, 1213; Ref. 48). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

anti-cancer therapy. Recently, Na et al.[46] reported the development of cancer-sensing MRI contrast agents (CR-CAs) using pH-responsive polymeric micelle. The pH sensitive CR-CAs self-assembled due to the amphiphilicity of the block copolymers consisting of methoxy poly(ethylene glycol)-*b*-poly(L-histidine) and methoxy poly(ethylene glycol)-*b*-poly(L-lactic acid)-diethylenetriaminopentaacetic acid dianhydride-gadolinium chelate. The CR-CAs showed a spherical shape with a uniform size of 40 nm at pH 7.4 (Figure 6). However, acidic tumor environment (pH 6.5) destabilized the CR-CAs by protonating the imidazole groups of p(His) blocks, causing them to break apart into positively charged water-soluble polymers. The CR-CAs were produced highly effective T_1 MR contrast enhancement in the acidic tumoral environment, enabling the detection of small tumors within minutes.

Farokhzad et al.[47] developed drug-encapsulated and pH responsive, surface charge-switching poly(D,L-lactic-co-glycolic acid)-*b*-poly(L-histidine)-*b*-poly(ethylene glycol) (PLGA-p(His)-PEG) nanoparticles for treating bacterial infections. These nanoparticle drug carriers were designed to shield nonspecific interactions at pH 7.4 and to bind specifically to bacteria in acidic conditions, delivering drugs and mitigating the loss of drug activity with declining pH. The mechanism involves pH-sensitive nanoparticle surface charge switching, which occurs as a result of selective protonation of the imidazole groups of p(His) at low pH. This PLGA- p(His)-PEG nanoparticle is useful as a drug carrier that can target and potentially treat gram-positive, gram-negative, or polymicrobial infections associated with acidity.

Torchilin et al. developed a pH-sensitive p(His)-PEG/DSPE-PEG mixed micelles for achieving quick intracellular drug release in response to the acidic pH of early endosomes. Higher intracellular drug concentrations were realized in this fashion. The micelles were internalized by receptor-mediated endocytosis, and the combination of active targeting and triggered release resulted in superior cytotoxicity and antitumor activity[48] (Figure 7). Therefore, multifunctional drug carriers combining the targeting ability and the stimuli-sensitivity show specific tumor cell targeting, enhanced cellular internalization, and rapid intracellular drug release.

In a recent contribution, we reported the development of a lipo-p(His) hybrid material, which showed pH-sensitivity, higher efficiency of intracellular delivery, and intrinsic tumor targetability.[49] Highly uniform Dox-loaded nanoassemblies fabricated from this new material showed good stability under physiological conditions. The Dox-loaded assemblies effectively suppressed MCF-7 tumors (Figure 8). A combination of the EPR effect, active internalization, endosomal pH-triggered release, escape of the drug from endosomes, and longer circulation time, all suggested that the IR-820 dye conjugated lipopeptide is a safe theranostic agent for imaging-guided cancer therapy.

The efficacy of nonviral gene therapy relies on the ability to deliver pDNA into the nucleus. Many of the newly developed delivery vehicles were built on existing designs in an attempt to improve efficacy. The cationic polymers of poly(L-lysine) (PLL) and p(His) possess membrane fusion capability.[50] The PLL-induced fusion occurs only outside the normal physiological pH conditions, whereas membrane fusion takes place at physiological pH when p(His) is used. The extent of p(His) fusion increased as the pH of the environment decreased.[51] Langer and coworkers developed a p(His)–PEG : DNA nanocomposite for gene delivery applications.[52] Two conjugate architectures (comb-shaped and linear A–B block copolymers) were complexed with plasmid DNA. The ability of the conjugates to form complexes with DNA, the hydrodynamic diameter, zeta potential, *in vitro* cytotoxicity, and transfection efficiency were determined. It was found that PEG content of the conjugate

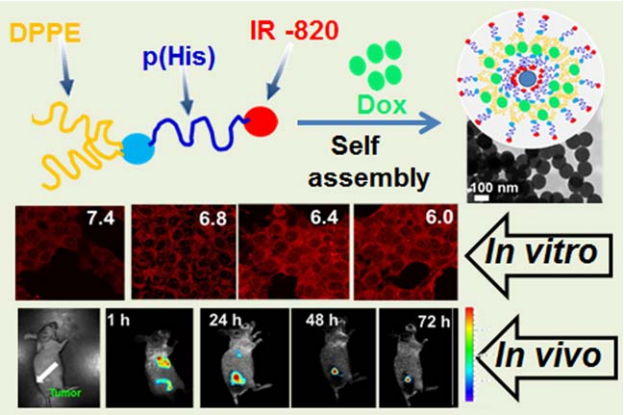

Figure 8. Diagrammatic representation of the pH dependent Dox release and *in vitro* and *in vivo* tumor imagining using the Dox loaded and IR-820 dye-conjugated micelles of lipo-poly(L-histidine) hybrid materials at tumor extracellular pH[49]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significantly influenced the hydrodynamic diameter of the DNA: conjugate complexes in aqueous solution. It was found that for comb-shaped conjugates, steric hindrance attributed to PEG led to a direct relationship between the PEG content and the complex size. Both architectures condensed plasmid DNA into nanostructures. The transfection efficiency was found to be approximately equivalent to DNA : PLL complexes. The low cytotoxicity of the complexes suggested that the system is useful for non-viral gene delivery.

Kim et al. designed a comb-shaped pH-sensitive cationic gene delivery vehicle composed of *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) polymer.[53] The *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) (p(His)-g-PLL) formed polyplex particles by way of electrostatic interactions with pDNA. The mean particle size of the polyplexes was found to be in the range 117–306 nm. The p(His)-*g*-PLL gene carrier produced higher transfection efficacy than PLL at all equivalent weight ratios with pDNA. Inclusion of chloroquine as an endosomolytic agent enhanced the transfection efficiency with both PLL and p(His)-*g*-PLL. These authors also developed the biodegradable delivery vector poly(ethylene glycol)-*co*-poly(L-lysine)-g-histidine for nonviral gene delivery.[54] *N*,*N*-dimethyl histidine was coupled at various mole ratios to the amines of PLL to produce PEG-PLL-g-His copolymer. The authors found that copolymers with 16% His produced the highest transfection efficiency with minimal cytotoxicity. Further, the polymers condensed pDNA into nanostructures with an average particle size between 150 nm and 200 nm and a surface charge of 4–45 mV. Overall, the results suggested that the biodegradable multiblock copolymers are promising gene delivery vectors.

Asayama et al. synthesized a p(His)-carbohydrate conjugate as a pH-sensitive drug carrier[55] and a p(His) with several aminoethyl groups as a pH-sensitive DNA carrier.[56] The p(His)-maltopentaose conjugate was synthesized by introducing aliphatic primary amino groups into p(His), followed by the reductive amination between the amino groups of p(His) and the reducing end of maltopentaose. The resulting p(His)-maltopentaose exhibited unique properties in solution at near physiological pH. By using the p(His)-maltopentaose as a pH-sensitive drug carrier, the assembling structure of the carrier–drug complex also varied around pH 6.[55] The authors examined if the pH of the medium influenced the complex formation with DNA, the cell membrane fusion activity, and the transfection efficiency. Agarose gel retardation assays showed that the polyion complex formation of the aminated p(His) with DNA was affected by pH owing to the basicity (protonation–deprotonation) of the imidazole groups that had a pK_a value of approximately 6.0. The DNA complexes enhanced the ability to disrupt membrane at endosomal pH. The aminated p(His) gene carrier produced good transfection efficacy. These results suggested that the aminated p(His) is a promising new pH-sensitive DNA carrier.[56]

Methylated p(His) has been evaluated for polyplex gene delivery.[57] From agarose gel retardation assay and zeta potential measurement showed that the anionic methylated p(His) coated the PEI/DNA binary complexes at physiological pH. The final methylated p(His) PEI/DNA ternary complexes produced 300 times higher gene expression than the PEI/DNA binary complexes. The effects of the pH-sensitive imidazole groups at endosomal pH and the anionic carboxymethyl groups at the physiological pH likely contributed to the enhanced polyplex gene delivery. Recently, these authors developed methylated p(His) with dimethylimidazole groups as a pH-sensitive polypeptide to control the stability of small interfering RNA (siRNA) polyion complexes used for RNA interference (RNAi).[58] It was found that the methylated p(His) was water-soluble despite the deprotonation of the imidazole groups at physiological pH. The quaternary dimethylimidazole groups functioned as cationic groups to retain siRNA. Further, the methylated p(His) exhibited no significant cytotoxicity in spite of the presence of cationic dimethylimidazole groups. When the methylated p(His) was used as a pH-sensitive siRNA carrier, the complexes mediated efficient siRNA delivery that was attributed to the dimethylimidazole groups.

POLY(HISTIDINE) CONTAINING DUAL RESPONSIVE INTRACELLULAR DELIVERY SYSTEMS

Bioconjugates of dual stimuli-responsive polymers are particularly interesting because their responsive nature can be conferred to the attached biological component.[59] Such materials show desirable properties such as thermo reversible aggregation and gelation, and have been used as biomaterials for variety of applications.[60] In the recent years, much effort has been focused on the synthesis of water soluble (co)polymers that undergo a conformational change or phase transition in response to external stimuli[61] such as temperature, added electrolyte, changes in pH, light, magnetic field, ultrasound, or a combination of stimuli. Among these stimuli, responsiveness to changes in temperature and pH are highly desirable for biomedical applications.

A number of pH-responsive micelles based on p(His) were discussed in the previous section. These micelles undergo structural destabilization at slightly acidic pH due to the protonation of p(His). However, designing sensitive pH-responsive micelles is challenging because of the subtle pH difference between the

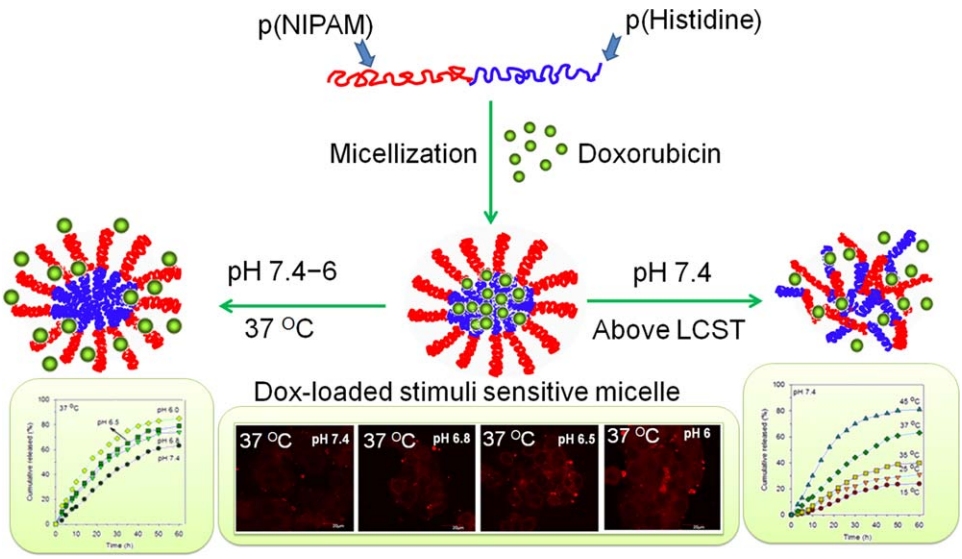

Figure 9. Diagrammatic representation of the fabrication of dual stimuli-responsive drug-loaded micelles prepared from p(NIPAM)-*b*-p(His) and the controlled delivery of Dox into HepG2 human hepatocellular carcinoma cells[18]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tumor and normal tissues. Advanced polymers with both thermo- and pH-responsive properties could circumvent these drawbacks. The dual-response properties of such copolymers would not only enable better on-demand drug release at the target site but also offer the synergistic effects of hyperthermia and chemotherapy. However, only limited number of reports are available on p(His) containing dual-stimuli responsive polymers. Yang et al. reported cooperative dual-stimuli-triggered aggregation of p(His) functionalized gold nanoparticles.[62] These authors found that gold nanoparticles functionalized with p(His) and exhibiting a pK_a value (~6.2) close to the physiological pH are sensitive to both pH and temperature. The pH and temperature must act in a cooperative way to trigger the conformational change of p(His) and the assembly/disassembly of the gold nanoparticles. The aggregation process and the conformation changes of p(His) could be recognized by the change in color of the gold nanoparticles. This cooperative dual-stimuli- triggered assembly/disassembly process of the gold nanoparticles may allow their potential applications in biosensing and biolabeling. We developed a synthetic polymer-polypeptide, poly(methyl methacrylate)-*b*-poly(L-histidine) (p(MMA)-*b*-p(His)), and utilized it for preparing a hybrid silver nanoparticle conjugate.[63] p(MMA)-*b*-p(His) was synthesized by combining ATRP of MMA and ROP of His-NCA. The resulting hybrid block copolymer formed reverse micelles in water and *N*,*N*-dimethylformamide (DMF), and self-assembled into p(His)/p(MMA) core/shell spheres with controllable size in the range 80–250 nm depending on the micellization temperature. The self-assembly of p(MMA)-*b*-p(His) was carried out in H_2O/DMF (3/7) mixture in the presence of $AgNO_3$. Reduction of the Ag ions encapsulated inside the reverse micelles yielded an attractive Ag nanoparticle core/polymer shell system.

In a more recent contribution, we reported the development of dual stimuli-responsive poly(*N*-isopropylacrylamide)-*b*-poly(L-histidine) (p(NIPAM)$_{55}$-*b*-p(His)$_n$) chimeric materials for the controlled delivery of Dox into liver carcinoma cells.[18] A series of p(NIPAM)$_{55}$-*b*-p(His)$_n$ ($n = 50$, 75, 100, 125) were synthesized by employing reversible addition−fragmentation chain transfer polymerization of NIPAM, followed by ROP of α-amino acid *N*-carboxyanhydrides. The dual stimuli-responsive properties of the resulting biocompatible and membranolytic p(NIPAM)$_{55}$-*b*-p(His)$_n$ polymers were investigated. Highly uniform self-assembled micelles (~55 nm) fabricated using p(NIPAM)$_{55}$-*b*-p(His)$_n$ polymers displayed sharp thermal and pH responses in aqueous media. Dox was effectively encapsulated in the micelles and the controlled Dox release was investigated at different temperature and pH conditions. The antitumor effect of the released Dox was also assessed using the HepG2 human hepatocellular carcinoma cells. Dox molecules released from the p(NIPAM)$_{55}$-*b*-p(His)$_n$ micelles remained biologically active and displayed stimuli-responsive killing of cancer cells (Figure 9). The ability of these hybrid materials to self-assemble into uniform micelles and the capacity of the resulting micelles to encapsulate Dox enable the use of these materials as drug carriers in cancer therapy. This block copolymer displays tunable properties that allow its use as molecular switching device for controlled drug delivery applications.

Ding et al.[64] developed *N*-Boc-Histidine-capped poly(D,L-lactide-*co*-glycolide)-poly(ethylene glycol)-poly(D,L-lactide-*co*-glycolide) (PLGA-PEG-PLGA) as a smart polymer for pH-sensitive drug delivery into tumors. The polymer was synthesized by introducing the *N*-Boc-histidine to the ends of a PLGA-PEG-PLGA block copolymer. This polymer was found to be biodegradable and biocompatible, and dissolved in water to form micelles above the critical micelle concentration. The release of Dox from the micelles of *N*-Boc-histidine capped PLGA-PEG-PLGA was different at pH 6.2 and pH 7.4, whereas Dox release from micelles made of uncapped polymers was not significantly sensitive to pH. The uptake of Dox from micelles of this polymer into MDA-MB-435 solid tumor cells was pH sensitive.

As an extension of the above work, Qiao et al. developed a thermo- and pH-responsive multiblock copolymer, poly(L-histidine)-poly(D,L-lactide-*co*-glycolide)-poly(ethylene glycol)-poly(D,L-lactide-*co*-glycolide)-poly(L-histidine) (p(His)-PLGA-PEG-PLGA-p(His)).[65] Initially, the PLGA-PEG-PLGA and p(His) were synthesized by ROP of DL-lactide and glycolide with PEG as an initiator and L-histidine *N*-carboxylanhydride with isopropylamine as an initiator. Finally, the copolymer was synthesized by the coupling reaction between p(His) and PLGA-PEG-PLGA. Following the construction of the copolymer micelles, the temperature- and pH-induced structural changes of the micelles were characterized by analyzing the particle size, the decrease in pyrene florescence intensity, and the changes in ^{1}H NMR spectra in D_2O. The hydrophobic–hydrophilic transitions of PEG and p(His) in response to temperature and pH changes accounted for the destabilization of the micelles. *In vitro* release profiles, as well as cell cytotoxicity and intracellular localization studies further confirmed the temperature- and pH- responsive properties of the copolymer micelles. These results demonstrate the potential of these copolymers to be used as stimuli-responsive carriers for targeted anti-cancer drug delivery.

CONCLUSIONS

Progress in polymer synthesis and cancer biology inspired researchers to develop smart nanovehicles for effective cancer treatment. Stimuli-responsive polymers have several advantages in drug delivery. Rather than acting passively as pure drug carriers, these materials interact and respond to the changes in the microenvironment. Generally, smart nanovehicles contain specific chains, ligands, or functional groups that are responsive to intrinsic or external stimuli, such as changes in pH, temperature, ultrasound, enzymes, or a combination of any of these factors. Taking advantage of the pH gradients within the tumor extracellular environments and in the intracellular compartments, pH sensitive micellar system have been developed for use in targeted drug delivery, which overcomes the limitations of conventional delivery systems. The p(His)-containing pH-sensitive polymeric micelles are a promising new class of programmable nano drug carriers that are likely to be used for targeted drug delivery in the near future.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid for the Fusion Research Program for Green Technologies (No. 2012M3C1A1054502) and the Basic Research Program (2012R1A1A204–1315) through the National Research Foundation of Korea funded by the Ministry of Education and the BK21 PLUS Program.

REFERENCES

1. Matsumura, Y.; Maeda, H.; *Cancer Res.* **1986**, *46*, 6387.
2. Maeda, H. *Adv. Enzyme Regul.* **2001**, *41*, 189.
3. Maeda, H.; Wu, J.; Sawa, T.; Matsumura Y.; Hori, K. *J. Control. Release.* **2000**, *65*, 271.
4. Kaneko, T.; Willner, D.; Monkovic, I.; Knipe, J. O.; Braslawsky, G. R.; Greenfield, R. S.; Vyas, D. M. *Bioconjugate Chem.* **1991**, *2*, 133.
5. Cammas, S.; Suzuki, K.; Sone, C.; Sakurai, Y.; Kataoka K.; Okano, T. *J. Control. Release* **1997**, *48*, 157.
6. Lee, E. S.; Na, K.; Bae, Y. H. *J. Control. Release* **2003**, *91*,103.
7. Lee, E. S.; Na, K.; Bae, Y. H. *Nano Lett.* **2005**, *5*, 325.
8. Lee, E. S.; Shin, H. J.; Na, K.; Bae, Y. H. *J. Control. Release* **2003**, *90*, 363.
9. Gao, Z. G.; Lee, D. H.; Kim, D. I.; Bae, Y. H. *J. Drug Target.* **2005**, *13*, 391.
10. Benns, J. M.; Choi, J. S.; Mahato, R. I.; Park, J. S.; Kim, S.W. *Bioconjugate Chem.* **2000**, *11*, 637.
11. Na, K.; Lee, E. S.; Bae, Y. H. *J. Control. Release* **2003**, *87*, 3.
12. Gillies, E. R.; Frechet, J. M. *Bioconjugate Chem.* **2005**, *16*, 361.
13. Bae, Y.; Nishiyama, N.; Fukushima, S.; Koyama, H.; Yasuhiro, M.; Kataoka, K. *Bioconjugate Chem.* **2005**, *16*, 122.
14. Kim, G. M.; Bae, Y. H.; Jo, W. H.; *Macromol. Biosci.* **2005**, *5*, 1118.
15. Sawant, R. M.; Hurley, J. P.; Salmaso, S.; Kale, A.; Tolcheva, E.; Levchenko, T. S.; Torchilin, V. P. *Bioconjugate Chem.* **2006**, *17*, 943.
16. Leeper, D. B.; Engin, K.; Thistlethwaite, A. J.; Hitchon, H. D.; Dover, J. D.; Li, D. J.; Tupchong, L.; *Int. J. Radiat. Oncol. Biol. Phys.* **1994**, *28*, 935.
17. Engin, K.; Leeper, D. B.; Cater, J. R.; Thistlethwaite, A. J.; Tupchong, L.; McFarlaneInt J. D.*J. Hypertherm.* **1995**, *11*, 211.
18. Johnson, R .P.; Jeong, Y. I.; Chung, John, J. V.; Chung, C. W., Kang, D. H.; Selvaraj, M.; Suh, H.; Kim, I. *Biomacromolecules* **2013**, *14*, 1434.
19. Gerasimov, O. V.; Boomer, J. A.; Qualls, M. M.; Thompson, D. H. *Adv. Drug Delivery Rev.* **1999**, *38*, 317.
20. Johnson, R. P.; Jeong, Y-I.; Chung, C. W.; Choi, E.; Kang, D. H.; Oh, S. O.; Suh, H.; Kim, I. *Adv. Funct. Mater.* **2012**, *22*, 1058.
21. Sethuraman V. A.; Bae, Y. H. *J. Control. Release* **2007**, *118*, 216.
22. Lee, E. S.; Na, K.; Bae, Y. H. *J. Control. Release* **2005**, *103*,405.
23. Schlossbauer, A.; Kecht, J.; Bein, T. *Angew. Chem. Int. Ed.* **2009**, *48*, 3092.
24. Schlossbauer, A.; Schaffert, D.; Kecht, J.; Wagner, E. *J. Am. Chem. Soc.* **2008**, *130*, 12558.
25. Sinha, V. R.; Kumria, R.; *Int. J. Pharm.* **2001**, *224*, 19.
26. Lee, J. W.; Park, J. H.; Robinson, J. R. *J. Pharm. Sci.* **2000**, *89*, 850.
27. Park, J. S.; Han, T. H.; Lee, K. Y.; Han, S. S.; Hwang, J. J.; Moon, D.H.; Kim, S. Y.; Cho, Y. W. *J. Control. Release* **2006**, *115*, 37.
28. Chen, A. Z.; Chen, M.Y.; Wang, S. B. Huang, X. N.; Liu, Y. G.; Chen, Z. X. *J. Appl. Polym. Sci.* **2012**, *124*, 3728.
29. Na, K.; Lee, E. S.; Bae, Y. H. *Bioconjugate Chem.* **2007**, *18*, 1568.
30. Wu, J-L.; Liu, C.; Wang, X.; Huang, Z. *J. Mater. Sci.- Mater. Med.* **2012**, *23*, 1921.

31. Qiu, L.; Li, Z.; Qiao, M.; Long, M.; Wang, M.; Zhang, X.; Tian, C.; Chen, D. *Acta Biomater.* **2013**, http://dx.doi.org/10.1016/j.actbio.2013.12.025.
32. Hwang, J-H.; Choi, C. W.; Kim, H-W.; Kim, D. H.; Kwak, T. W.; Lee, H. M.; Kim, C. H.; Chung, C.W, Jeong, Y-I.; Kang, D. H. *Int. J. Nanomedicine* **2013**, *8*, 3197.
33. Thomas J. J.; Rekha, M. R.; Sharma, C. P. *Mol. Pharmaceutics* **2012**, *9*, 121.
34. Oh, K. T.; Yin, H.; Lee, E. S.; Bae, Y. H. *J. Mater. Chem.* **2007**, *17*, 3987.
35. Lee, E. S; Gao, Z.; Kim, D.; Park, K.; Kwon I. C.; Bae, Y. H. *J. Control. Release* **2008**, *129*, 228.
36. Liu, Y.; Wang, W.; Yang, J.; Zhou, C.; Sun, J. *Asian J. Pharm. Sci.* **2013**, *8*, 159.
37. Oh, K. T., Lee, E. S., Kim, D., Bae, Y. H. *Int. J. Pharm.* **2008**, *358*, 177.
38. Kim, D.; Lee, E. S.; Park, K.; Kwon, I. C, Bae, Y. H. *Pharm. Res.* **2008**, *25*, 2074.
39. Kim, D.; Lee, E. S.; Oh, K. T., Gao, Z .G.; Bae, Y. H. *Small* **2008**, *4*, 2043.
40. Kim, D.; Gao, Z .G, Lee, E. S.; Bae, Y. H. *Mol. Pharmaceutics* **2009**, *6*, 1353.
41. Johnson, R. P.; Choi, E.; Lee, K. M.; Yu, S. J. Chang, H. C.; Suh, H.; Kim, I. *Microfluid. Nanofluid.* **2013**, *14*, 257.
42. Lee, Y. J.; Kang, H. C.; Hu, J.; Nichols, J. W.; Jeon, Y. S, Bae, Y. H. *Biomacromolecules* **2012**, *13*, 2945.
43. Park, W.; Kim, D.; Kang, H C.; Bae, Y. H., Na, K. *Biomaterials* **2012**, *33*, 8848.
44. Johnson, R. P.; Chung, C. W.; Jeong, Y-I.; Kang, D. H.; Suh, H.; Kim, I. *Int. J. Nanomedicine* **2012**, *7*, 2497.
45. Hu, J.; Miura, S.; Na, K.; Bae, Y. H. *J. Control. Release* **2013**, *172*, 69.
46. Kim, K. S.; Park, W.; Hu, J.; Bae, Y. H., Na, K. *Biomaterials* **2014**, *35*, 337.
47. Radovic-Moreno, A. F.; Lu, T. K.; Puscasu, V. A.; Yoon, C. J.; Langer, R.; Farokhzad, O. C. *ACS Nano* **2012**, *6*, 4279.
48. Wu, H.; Zhu, L.; Torchilin, V. P. *Biomaterials* **2013**, *34*, 1213.
49. Johnson, R. P.; Jeong, Y-I.; John, J. V.; Chung, C. W.; Choi, S. H.; Song, Y. S.; Kang, D. H.; Suh, H.; Kim, I. *Macromol. Rapid Commun.* **2014**, DOI: 10.1002/marc.201300892.
50. Uster, P. S.; Deamer, D. W. *Biochemistry* **1985**, *24*, 1.
51. Wang, C. Y.; Huang, L. *Biochemistry* **1984**, *23*, 4409.
52. Putnam, D.; Zelikin, A. N.; Izumrudov, V. A, Langer, R. *Biomaterials* **2003**, *24*, 4425.
53. Benns, J. M.; Choi, J-S.; Mahato, R. I.; Park, J-S.; Kim, S. W. *Bioconjugate Chem.* **2000**, *11*, 637.
54. Bikram, M.; Ahn, C-H.; Chae, S. Y.; Lee, M.; Yockman, J. W.; Kim, S. W. *Macromolecules* **2004**, *37*, 1903.
55. Asayama, S.; Kawakami, H.; Nagaoka, S. *Polym. Adv. Technol.* **2004**, *15*, 439.
56. Asayama, S.; Sekine,T.; Hamaya, A.; Kawakami, H.; Nagaoka, S. *Polym. Adv. Technol.* **2005**, *16*, 567.
57. Asayama, S.; Sudo, M.; Nagaoka, S. Kawakami, H.; *Mol. Pharmaceutics* **2008**, *5*, 898.
58. Asayama, S.; Kumagai, T.; Kawakami, H. *Bioconjugate Chem.* **2012**, *23*, 1437.
59. McCormick, C. L.; Sumerlin, B. S.; Lokitz, B. S.; Stempka, J. E. *Soft Matter* **2008**, *9*, 1760.
60. Schmaljohann, D. *Adv. Drug Delivery Rev.* **2006**, *58*, 1655.
61. Zhang, J.; Pelton, R.; Deng, Y. *Langmuir* **1995**, *11*, 2301.
62. Guan, J.; Li, J.; Guo, Y.; Yang, W. *Langmuir* **2009**, *25*, 2679.
63. Shin, N. H.; Lee, J. K.; Li, H.; Ha, C-S.; Shchipunov, Y. A.; Kim I. *J. Nanosci. Nanotechnol. 10*, **2010**, 1.
64. Chang, G.; Li, C.; Lu, W.; Ding, J. *Macromol. Biosci.* **2010**, *10*, 1248.
65. Hong, W.; Chen, D.; Jia, L.; Gu, J.; Hu, H.; Zhao, X.; Qiao, M. *Acta Biomater.* **2014** *10*, 1259.