Thermally Responsive Collagen Switching from Bactericidal for Ambient Storage Condition to Biologically Inert *in Vivo*

by

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Abstract

Incorporating preservatives is now the most commonly employed strategy to prevent microbial deterioration of collagen. However, many preservatives are non-selective; they cannot differentiate the orthologs in mammalian cells from their biological targets in pathogens, and thus their incorporation compromises the inherent biocompatibility of naturally occurring collagen. Here, we report a strategy to covalently anchor antibacterial agents to collagen *via* a thermally responsive poly(*N*-isopropylacrylamide) spacer. As the spacer suddenly collapses from an extended coil upon heating through ~30°C, bioactivity of the anchored antibacterial agents can be switched off, because their lateral mobility and, hence, target-site accessibility are restricted. Accordingly, the modified collagen can be preserved without microbial deterioration for ambient storage condition, while imposing no cytotoxic impact upon surrounding tissues *in vivo*. This thermally responsive collagen holds great potential in biomedical fields, where the collagen products are intended to have intimate contact with human tissues or be administered parenterally.

Introduction

Collagen is the most abundant protein in mammals, making up from 25 to 35% of the whole-body protein content. After being secreted by the cells, the collagen molecules assemble into fibers of unique structure, responsible for structural integrity and physiological functions of tissues. A single collagen molecule, or tropocollagen, is composed of three polypeptide strands, each of which has the conformation of a left-handed helix. These three helices are then twisted together into a right-handed triple helix, a cooperative quaternary structure stabilized by hydrogen bonds. Owing to excellent anti-inflammatory property,[1] low antigenicity,[2] and non-cytotoxicity,[3] this fibrous protein has currently been extensively used, particularly in the fields of pharmaceutical and biomedical engineering.[4]

However, collagen is prone to microbial colonization, which leads to collagen degradation by microbial enzymes, changes in impurity profiles, as well as an increase in bacterial endotoxins.[5,6] Hence, collagen must be subject to preservative treatment immediately after extracted from tissues, preventing microbial deterioration before further processing or end use. The most commonly employed strategy for collagen preservation nowadays is incorporation of low-molecular-weight preservatives, including propylparaben,[7] chloroxylenol,[8] sodium benzoate,[9] potassium sorbate,[10] *etc*. Despite the efficiency of this approach, most of these preservatives are non-selective. They cannot distinguish the orthologs in mammalian cells from their biological targets in pathogens, and thus their incorporation may cause cytotoxic or allergic effects, compromising the inherent biocompatibility of naturally occurring collagen. For example, propylparaben can irritate eyes, skin, intestinal, and respiratory tracts upon contact, ingestion, and inhalation.[11]

In addition, cases of allergic contact dermatitis caused by chloroxylenol have also been extensively reported, while acute oral poisoning by chloroxylenol-containing formulations has proved fatal.[12] Such dilemma is the case particularly for those collagen products intended to have intimate and continuous contact with human tissues. Ideally, a collagen product should exhibit antibacterial properties for ambient storage condition, while turning biologically inert, or biocompatible, for *in vivo* condition. This feature is extremely attractive for collagen products that have to be ultimately introduced into living beings. Here, we report synthesis of a thermally sensitive poly(*N*-isopropylacrylamide) (PNIPAM) chain with a ciprofloxacin antibacterial moiety at one terminal and an *N*-hydroxysuccinimide (NHS) ester at the other by reversible

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addition-fragmentation chain-transfer (RAFT) polymerization. The NHS ester can react preferentially with primary amines under mild condition, enabling covalent anchor of the conjugate onto naturally occurring collagen. Upon heating through the lower critical solution temperature (LCST, ~30°C) of the PNIPAM spacer, it is found that the antibacterial potency of the modified collagen against Gram-negative bacteria can be switched off. This phenomenon was ascribed to temperature-triggered coil-to-globule transition of the PNIPAM spacer, which regulated lateral mobility and, hence, target-site accessibility of the ciprofloxacin terminal. The modified collagen can then be preserved for ambient storage condition without microbial deterioration, while displaying no preservatives-associated cytotoxicity *in vivo*. This feature should be practically useful in biomedical fields, where the collagen products are intended to have intimate contact with human tissues or be administered parenterally. To the best of our knowledge, there are no existing literatures as yet reporting on a similar subject to that presented herein.

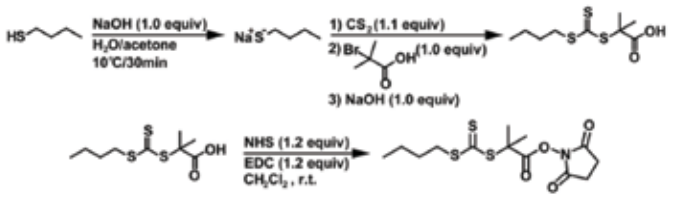
Experimental Procedures

Materials

Type I collagen powder from bovine skin, ciprofloxacin (≥98.0%), acryloyl chloride (97.0%), triethylamine (Et_3N, ≥99.0%), carbon disulfide (anhydrous, ≥99.0%), 2-methyl 2-bromopropanoic acid (≥99.0%), 1-butanethiol (99.0%), 2,2'-azobis(2-methylpropionitrile) (AIBN, 98.0%), and *N*-isopropylacrylamide (NIPAM, ≥99%) were purchased from Sigma-Aldrich (Shanghai, China). The NIPAM monomers were recrystallized twice from toluene/*n*-hexane (10:90 *v/v*) before use. *N*-hydroxysuccinimide (NHS, ≥98.0%) and 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride (EDC·HCl, ≥98.5%) were obtained from Alfa Aesar (Ward Hill, MA). LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (L-7012) containing SYTO 9 dye and propidium iodide (PI) for microscopy was acquired from Thermo Fisher Scientific, Inc. (Waltham, MA).

Synthesis of NHS Ester-Activated RAFT Agent

The RAFT agent, 2-(((butylthio)carbonothioyl)thio)-2-methylpropanoic acid, was prepared according to a previously disclosed procedure (Scheme 1).[13] In brief, a 20 wt % aqueous solution of sodium hydroxide (13.7 g) was added into a flask equipped with a nitrogen inlet and a reflux condenser, which was pre-charged with a solution of 1-butanethiol (68.85 mmol) in acetone (5 mL). Under nitrogen atmosphere, the mixture was stirred at 10°C for 30 min, and then 75.74 mmol carbon disulfide was added *via* cannula over 15 min. After stirred for another 30 min, 68.85 mmol 2-methyl 2-bromopropanoic acid was added slowly. Then, a 20 wt % aqueous solution of sodium hydroxide (13.7 g) was added slowly such that the temperature did not exceed 30°C. Subsequently, the orange solution was stirred at room temperature for 24 h, and then poured into 50 mL ultrapure water. The mixture was neutralized by HCl, and the product was extracted using dichloromethane. The organic phase was dried over anhydrous magnesium sulfate, filtered, and then subject to rotary evaporation, resulting in 2-(((butylthio) carbonothioyl)thio)-2-methylpropanoic acid with a yield of 68%.



Scheme 1. Synthesis procedure of NHS ester-activated RAFT agent.

The carboxyl in the RAFT agent synthesized above was activated using NHS/EDC chemistry (Scheme 1). EDC.HCl (2.0 g, 10.37 mmol) and NHS (1.2 g, 10.37 mmol) were added into a solution of 2-(((butylthio)carbonothioyl)thio)-2-methylpropanoic acid (2.2 g, 8.64 mmol) in anhydrous dichloromethane (30 mL). The mixture was stirred at room temperature for 18 h and diluted with 20 mL dichloromethane. Then, the solution was sequentially washed with water, brine, dried over Na_2SO_4 and filtered. Removal of the solvent under vacuum yielded the NHS ester-activated RAFT agent as a yellow powder. Chemical structure of the product was confirmed by nuclear magnetic resonance (NMR) analysis (Figure 1).

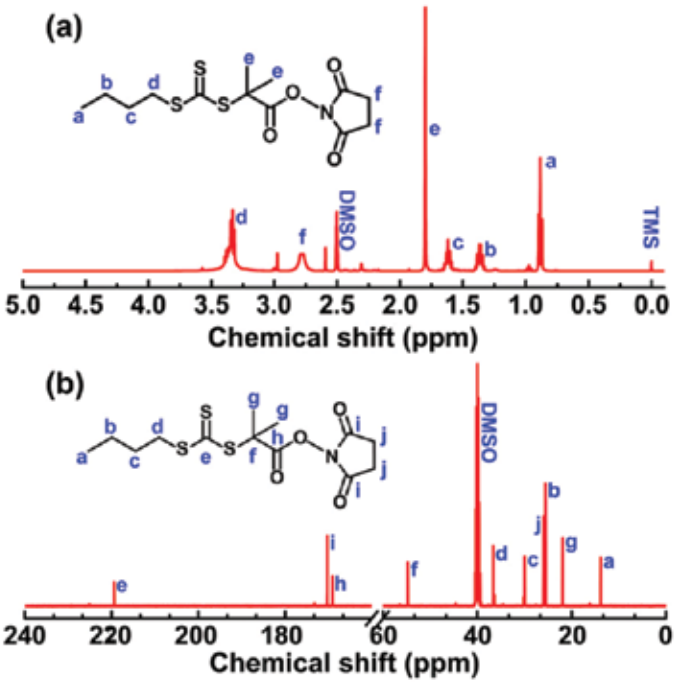
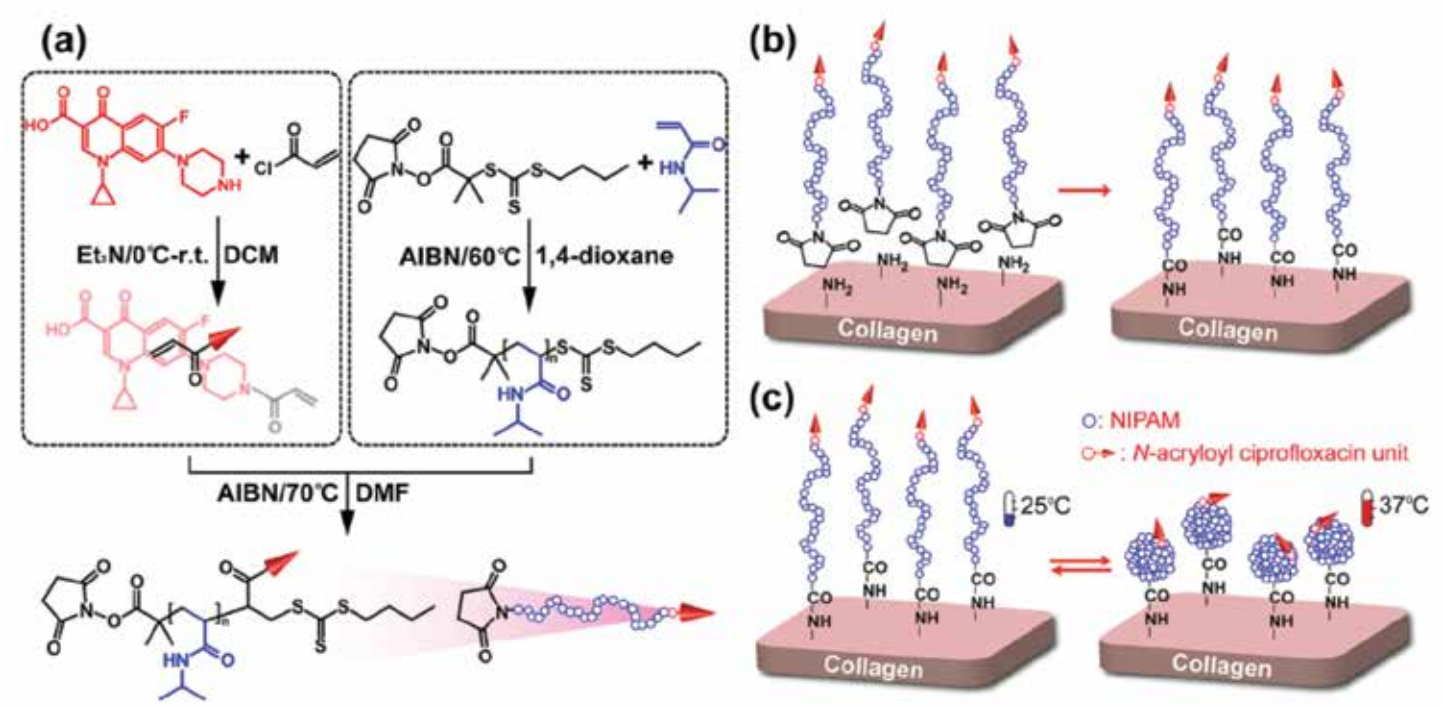


Figure 1. (a) ^1H and (b) ^{13}C NMR spectra of NHS ester-activated RAFT agent.



Scheme 2. (a) Synthesis procedure of PNIPAM-ciprofloxacin conjugate. (b) Covalent anchoring of PNIPAM-ciprofloxacin conjugate to collagen *via* NHS ester-mediated acylation. (c) Schematic illustration of how temperature influences lateral mobility of the ciprofloxacin terminal in the PNIPAM-ciprofloxacin conjugate covalently anchored on collagen.

Synthesis of *N*-acryloyl Ciprofloxacin

A polymerizable double bond was introduced into ciprofloxacin by acryloylation of the secondary amino group in ciprofloxacin with acryloyl chloride (Scheme 2 (a)). Detailed procedure for this reaction was disclosed in our recent publications.[6, 14, 15]

Synthesis of PNIPAM-Ciprofloxacin Conjugate

PNIPAM-ciprofloxacin conjugate was synthesized *via* sequential solution RAFT polymerization (Scheme 2 (a)). NIPAM (4.8 g, 42.24 mmol), NHS ester-activated RAFT agent (153 mg, 0.44 mmol), AIBN (7 mg, 0.04 mmol), and 1,4-dioxane (14.0 g) were added into a 50 mL Schlenk flask. The mixture was degassed *via* three cycles of freezing and thawing, and then heated up to 60°C. After 4 h, the polymerization was quenched by rapid cooling upon immersion of the flask into iced water. The resulting PNIPAM product was precipitated into cold diethyl ether. The precipitate was collected by filtration and then re-dissolved into 1,4-dioxane. This precipitation-filtration procedure was repeated three times. The precipitate was finally dried at room temperature under vacuum overnight to afford a yellow powder (3.1 g, 63% yield).

Subsequently, *N*-acryloyl ciprofloxacin (0.6 g, 1.56 mmol), PNIPAM prepared above (3.0 g, 0.31 mmol), and AIBN (10.0 mg, 0.06 mmol) were mixed in 12.0 g *N,N*-dimethylformamide. The mixture was degassed *via* three cycles of freezing and thawing, and then stirred at 70°C for 4 h. After that, the polymerization was quenched by immersing the flask into iced water. The mixture was then precipitated into cold water and filtered. The filtrate was dried under vacuum at room temperature until a constant weight, followed by being re-dissolved into *N,N*-dimethylformamide. The precipitation-filtration procedure was repeated three times. Finally, the yellow product was dissolved into distilled water, and purified by preparative high performance liquid chromatography on an Agilent binary pump system using a YMC-Pack Pro C18 column. The sample was eluted with a mobile phase containing acetonitrile and water in a ratio of 20:80 (*v/v*) at a flow rate of 5.0 mL/min and monitored at dual wavelength of 210 and 273 nm for PNIPAM and ciprofloxacin, respectively. Upon lyophilization, a yellow powder with a yield of 58% could be obtained. The synthesis procedure mentioned above is depicted in Scheme 2 (a).

Covalent anchoring of PNIPAM-Ciprofloxacin Conjugate onto Collagen

Native collagen powder was first dissolved in 0.5 M aqueous solution of acetic acid, yielding a 5 mg/mL solution. The solution was then cast to form a membrane, which was dried under vacuum at 37°C until a constant weight. The resulting collagen membrane was immersed in 100 mL of a phosphate buffer saline (PBS) solution containing 30 wt% PNIPAM-ciprofloxacin conjugate overnight. During this process, NHS ester-

mediated acylation between the conjugate and the collagen occurred, as depicted in Scheme 2 (b). After that, the collagen membrane was rinsed in PBS for another 24 h and washed with deionized water five times. The modified collagen membrane was dried at 37°C under vacuum and then stored at 4°C before use.

Characterization

NMR spectra were recorded on a Bruker TD-65536 NMR (400 MHz) instrument using deuterated dimethyl sulfoxide (DMSO-d_6, 99.9 atom% D) containing 0.03% (*v/v*) tetramethylsilane (TMS) as the solvent. Size-exclusion chromatography (SEC) was conducted on a Waters 2690D separation module equipped with a TSK-GEL column and a Waters 2410 refractive index detector. Tetrahydrofuran containing 3 wt% triethylamine with a flow rate of 0.5 mL/min was employed as the eluent. X-ray photoelectron spectroscopy (XPS) analysis was carried out on an AXIS Ultra DLD instrument (Kratos, U.K.), equipped with a standard and monochromatic Al Kα X-ray excitation source (1486.6 eV). The binding energies were corrected by referencing the C 1s peak at 284.6 eV. Fourier transform infrared spectroscopy (FT-IR) analysis was performed on a Nicolet iS10 FT-IR spectrometer (Thermo Scientific, U.S.) over a wavenumber range from 600 to 3000 cm^{-1} after 64 scans at a resolution of 2 cm^{-1}. Turbidimetry was conducted on a UV-vis spectrophotometer (UV-3600, SHIMADZU, Japan). The aqueous sample solution (concentration=1.0 g/L) was loaded in a quartz cuvette of 10 mm path length and heated at a rate of 0.2°C/min. Circular dichroism (CD) spectra were obtained at 25°C under a nitrogen atmosphere using an AVIV model 400 CD spectrometer (AVIV Biomedical, Inc., US).

Escherichia coli (*E. coli*, ATCC 25922) was chosen as the indicator to evaluate the antibacterial potency of the collagen modified with PNIPAM-ciprofloxacin conjugate. The strain was cultured in Müller-Hinton broth at 37°C for 12 h, which was then harvested by centrifugation and washed with PBS three times. Subsequently, a suspension of *E. coli* in PBS (1×10^5 CFU/mL, 50 μL) was inoculated on the modified collagen surface and incubated at different temperatures for 24 h. After that, the sample was washed with PBS three times, and soaked in 8 mL of a dye solution containing 4 μL SYTO 9 and 6 μL PI at room temperature in dark for 15 min. The stained bacteria were observed using an IX-71 Inversed Fluorescent Microscope (Olympus America Inc., Melville, NY). *Image J* (NIH, Bethesda, US) software was utilized to process the fluorescent images obtained above.

Cell proliferation assay was conducted according to ISO 10993-5 to evaluate the cytocompatibility of the modified collagen. Detailed procedure for this experiment was disclosed in our recent publication.[6]

Results and Discussion

To attach ciprofloxacin to only one terminal of the PNIPAM spacer, RAFT polymerization technique, allowing chain extension of a polymer of one monomer with a second monomer, was used to synthesize the conjugate. The RAFT agent employed in this study possessed a carboxyl terminal, which was activated by NHS/EDC chemistry before polymerization. The NHS ester-activated carboxyl was designed to react with those primary amino group provided by collagen under mild condition, yielding a stable amide linkage that anchored the conjugate onto naturally occurring collagen. Using a mixture of monomer, initiator, and NHS ester-activated RAFT agent of carefully designed compositions, a narrow-polydispersed PNIPAM (M_n=9560 g/mol; M_w=10114 g/mol; PDI=1.06) was synthesized, which was constituted by approximately 85 NIPAM monomeric units. Furthermore, retention of a thiocarbonylthio cap in the PNIPAM product enabled consecutive polymerization of vinyl ciprofloxacin monomers from only one end of the PNIPAM chain, yielding the final conjugate. To this end, ciprofloxacin, a synthetic broad-spectrum antibacterial agent, was acryloylated by site-specific reaction of the secondary amino group in the 7-piperazinyl substitute with acryloyl chloride. The parent ciprofloxacin operates by hydrogen-bonding with DNA gyrase and topoisomerase IV in bacteria, trapping these enzymes at the DNA cleavage stage to prevent strand rejoining.[16] To approach their intracellular targets, ciprofloxacin is known to utilize the non-specific aqueous porin channels that span the entirety of the outer membrane of Gram-negative bacteria for translocation. Recently, we showed that the as-synthesized *N*-acryloyl ciprofloxacin was still bactericidal, because the 3-carboxyl, 4-keto, and quinolone pharmacophore, all essential for hydrogen-bonding with DNA gyrase and topoisomerase IV to inhibit *in vivo* DNA synthesis, remained intact.[6, 14, 15] By consecutive RAFT polymerization, the antibacterial ciprofloxacin was attached to one end of the PNIPAM chain *via* noncleavable linkages. According to the ^{1}H NMR spectrum illustrated in Figure 2 (a), three un-overlapped resonance signals in the range of 7.9-8.9 ppm (indicated by red inverted triangle) were detected in the PNIPAM-ciprofloxacin conjugate, ascribed to specific protons in ciprofloxacin moiety.[17] This observation indicated successful conjugation of *N*-acryloyl ciprofloxacin to the PNIPAM spacer. In addition, another resonance signal peaking at 2.8 ppm appeared, which was associated with NHS moiety. Based on this NMR spectrum, the average number of *N*-acryloyl ciprofloxacin monomeric units per conjugate chain was calculated to be 0.8, consistent with the result from pyrolysis-gas chromatography/mass spectrometry, indicating a ciprofloxacin content of *ca.* 1.1 mol%.

With the ciprofloxacin and the NHS-ester terminals, the PNIPAM spacer remained thermally sensitive, experiencing the

well-known coil-to-globule transition upon heating, manifested as a sudden change of transmittance with increasing temperature, as recorded by turbidimetry. Compared with pure PNIPAM with a LCST of 32.0°C, single end-capping with a limited number of vinyl ciprofloxacin monomers slightly lowered the temperature where the coil-to-globule transition occurred, with the LCST of the conjugate detected at 30.2°C (Figure 2 (b)).

With an NHS-ester terminal, the conjugate could be covalently anchored onto naturally occurring collagen *via* NHS ester-mediated acylation. Successful anchoring of PNIPAM-ciprofloxacin conjugate on type I collagen surface was qualitatively verified by XPS analysis. According to Figure 2 (c), an XPS signal for F 1s at 688.1 eV was detected on the modified collagen, corresponding to the 6-fluoro substitute in ciprofloxacin. Then, the activity of the modified collagen against Gram-negative *E. coli* was evaluated by LIVE/DEAD *Bac*Light bacterial viability kit at 25°C, well below the LCST of the PNIPAM spacer, and at physiological temperature 37°C. This viability assay utilizes mixtures of SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, PI. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population-those with intact membranes and those with damaged membranes. In contrast, PI penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and PI stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red.[18, 19] As could be seen in Figure 3, the modified collagen displayed quite different, or switchable, antibacterial potency against *E. coli* in response to temperature. First, the modified collagen was bactericidal at 25°C, and hence the overwhelming majority of the bacteria attached on the collagen surface fluoresced red. According to this observation, it seemed that the ciprofloxacin terminals, although covalently anchored on collagen, were still able to approach their intracellular targets when the PNIPAM spacer extended. This was because the PNIPAM spacer designed in this study was constituted by approximately 85 NIPAM monomeric units, corresponding to a contour length of *ca.* 21.3 nm. When the ciprofloxacin terminals diffused through the porin channels for internalization, the long PNIPAM spacer would allow them to access cytoplasm by traversing the whole cell envelope of *E.coli*, which possessed an average thickness of *ca.* 16 nm.[20] Upon heating to 37°C, the PNIPAM spacer collapsed into a globule, preventing lateral diffusion of the ciprofloxacin terminals. Since the ciprofloxacin terminals were confined to the collagen surface in this case, they could not diffuse through the porin channels in the outer membrane of *E. coli*, and therefore, the modified collagen turned into biologically inert as if the PNIPAM-ciprofloxacin conjugate

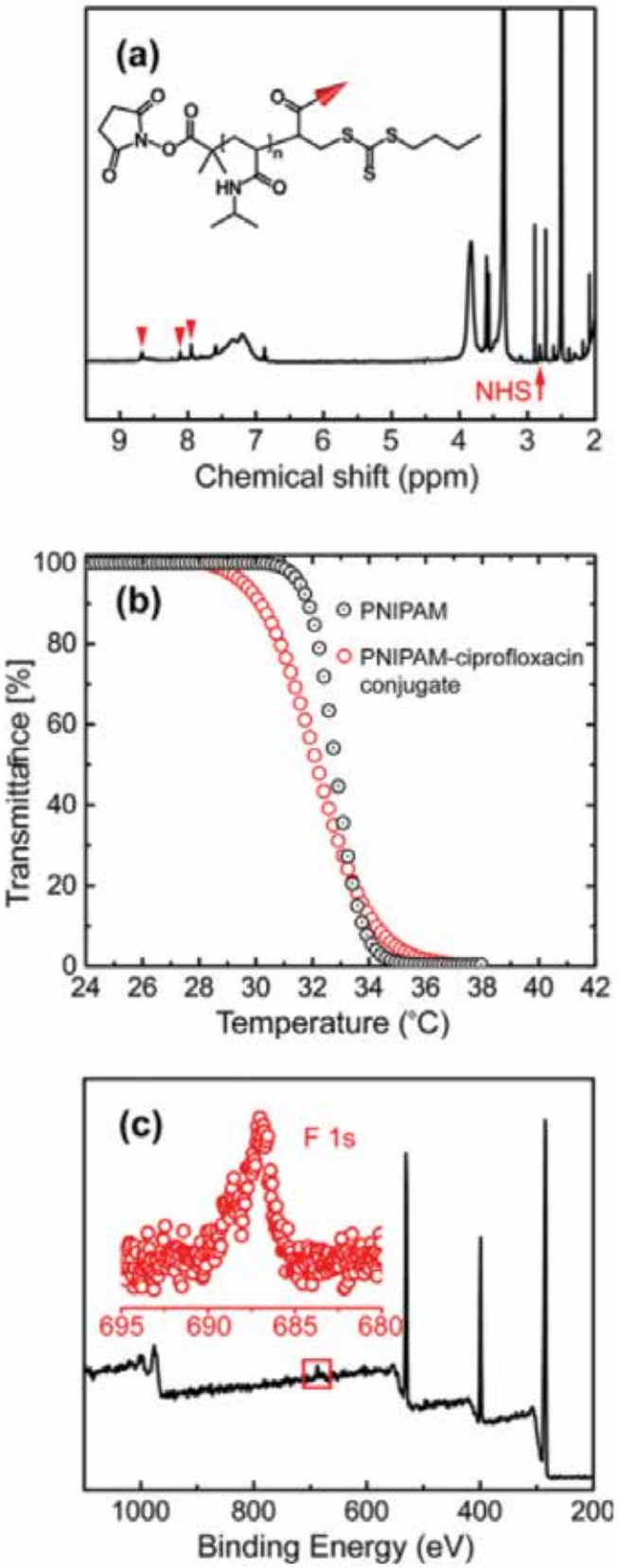


Figure 2. (a) ^{1}H NMR of PNIPAM-ciprofloxacin conjugate. (b) Changes in transmittance with increasing temperature for aqueous solution of PNIPAM-ciprofloxacin conjugate. LCST was determined as the temperature of the solution corresponding to 90% transmittance. (c) XPS survey spectrum of collagen modified by PNIPAM-ciprofloxacin conjugate.

were not there. Consequently, the bacteria inoculated onto the modified collagen were viable and fluoresced green as shown in Figure 3. According to these results, the modified collagen was bactericidal at low temperature, capable of preventing microbial deterioration for ambient storage condition. Upon introducing *in vivo*, the PNIPAM spacer collapsed at physiological temperature, which switched off the bioactivity of the anchored ciprofloxacin, restoring the inherent biocompatibility of the modified collagen.

Furthermore, FT-IR and CD experiments were conducted to determine whether the modification by PNIPAM-ciprofloxacin conjugate damaged the special triple helix of tropocollagen, which is considered as the foundation of many biological functions of collagen. FT-IR spectra of collagen before and after modification are displayed in Figure 4 (a) for comparison. Generally speaking, the intensity ratio of FT-IR absorption at 1235 to that at 1450 cm^{-1} ($I_{1235/1450cm^{-1}}$) can be used as a sensitive indicator for the integrity of the triple-helical structure in collagen.[21, 22] According to Figure 4 (a), the $I_{1235/1450cm^{-1}}$ of the modified collagen was determined to be 0.97, comparable to that of the native collagen (1.02). This result suggested that the modification by PNIPAM-ciprofloxacin conjugate imposed almost no influence on the triple-helical conformation of the collagen.

In addition, CD analysis provided similar results. According to previous reports,[23, 24] the absolute ratio of molar ellipticity at 223 nm to that at 197 nm (Rpn) was correlated with the integrity of the triple-helical conformation of collagen in solution. In Figure 4 (b), the CD spectrum of native collagen exhibited a negative peak at approximately 197 nm and a positive peak at around 223 nm, suggesting a typical triple-helical conformation. Once modified by PNIPAM-ciprofloxacin conjugate, the CD spectrum hardly changed compared with the native one, indicating preservation of the triple helicity. Particularly, the Rnp value (0.141) of the modified collagen was found almost identical to that of the native one (0.137), suggesting that modification by PNIPAM-ciprofloxacin conjugate barely damaged the triple helix of tropocollagen.

Once the PNIPAM spacer collapsed, enabling confinement of the ciprofloxacin terminals, the modified collagen exhibited biocompatibility as good as the native one. The morphologies of NHDFs cultured in collagen-coated PS wells for three days are shown in Figure 5 (b_1) and (b_2). It could be seen that the cells on the modified collagen displayed almost the same morphologies to those on native collagen, showing typical fibroblast morphologies. This observation suggested that the modified

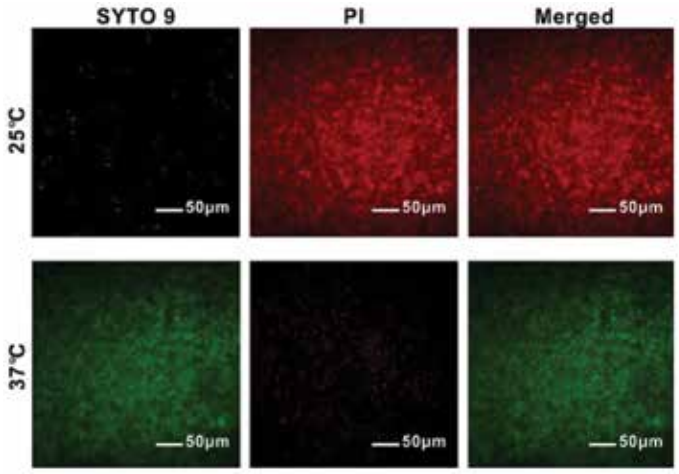


Figure 3. Fluorescent images illustrating *E. coli* on the surface of collagen modified with PNIPAM-ciprofloxacin conjugate. The green fluorescence indicates live bacteria, while the red represents dead bacteria. The merged images represent the superposition of live and dead bacteria.

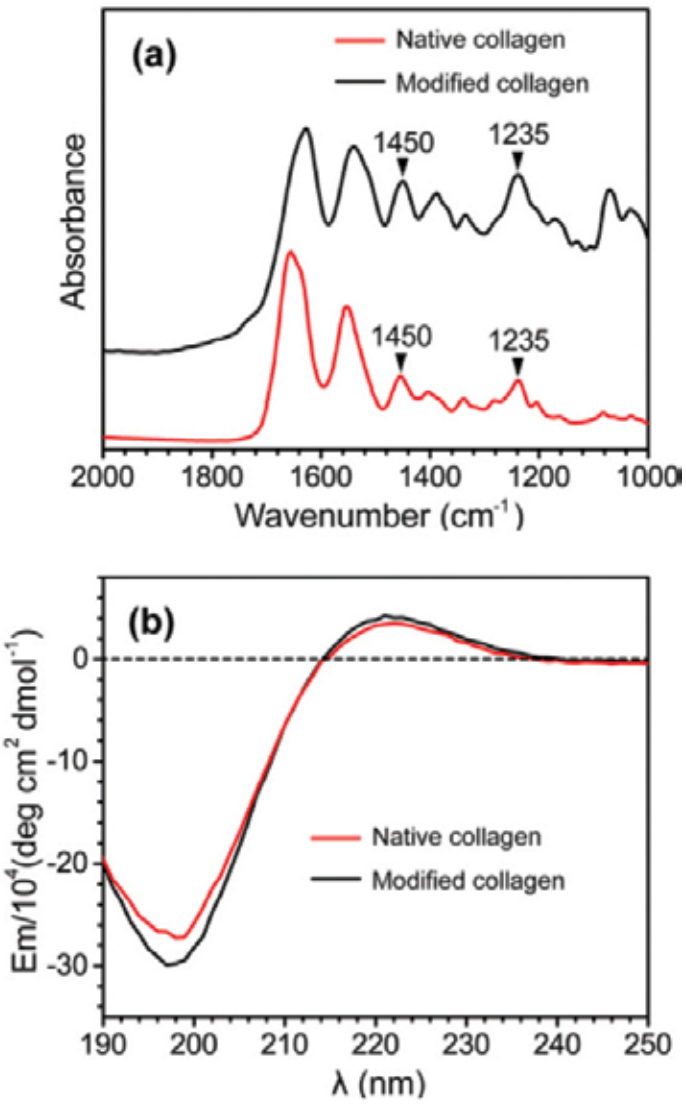


Figure 4. (a) FT-IR spectra and (b) CD spectra of native collagen and collagen modified by PNIPAM-ciprofloxacin conjugate.

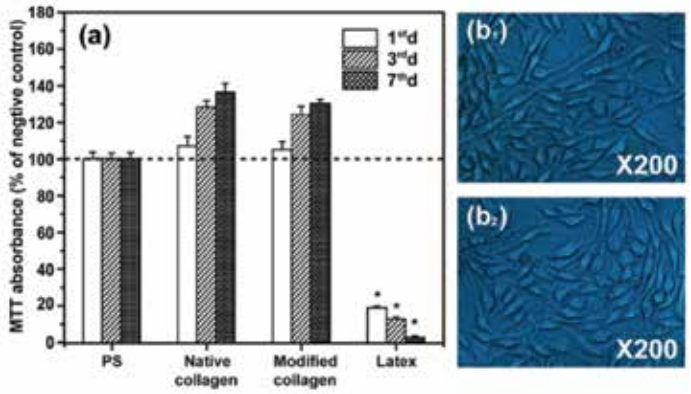


Figure 5. (a) Proliferation rate of NHDFs on collagen-coated PS wells with increasing time. Latex was used as positive control. At each time interval, the MTT absorbance was normalized with that of negative control (un-coated PS wells). Error bars represent means±standard deviation for n=3. Statistical significance: $*p < 0.05$. Morphologies of NHDFs on PS wells pre-coated with (b_1) native collagen, and (b_2) modified collagen after seeded for three days.

collagen supported differentiation of NHDFs as effectively as the native one. Furthermore, the proliferation rate of NHDFs was measured by using MTT assay. As illustrated in Figure 5 (a), the NHDFs on the modified collagen proliferated faster than those on the uncoated PS wells for the same culturing time. Nevertheless, the MTT absorbance of positive control (latex) was significantly low regardless of time, because the overwhelming majority of cells were killed by toxic materials released from latex.

Conclusions

A thermally sensitive PNIPAM chain with an antibacterial ciprofloxacin moiety at one terminal and an NHS ester at the other could be successfully synthesized by RAFT polymerization. This conjugate could be covalently anchored onto naturally occurring collagen *via* NHS ester-mediated acylation. The modified collagen was bactericidal below the LCST of the PNIPAM spacer, but the bioactivity of the ciprofloxacin terminal could be switched off upon heating up to physiological temperature. This phenomenon was due to temperature-triggered coil-to-globule transition of the PNIPAM spacer, which regulated lateral mobility and, hence, target-site accessibility of the ciprofloxacin terminal. Thus, the modified collagen could be preserved without microbial deterioration for ambient storage condition while exhibiting no cytotoxic impact upon surrounding tissues *in vivo*. The thermally responsive collagen reported herein may be of great potential for applications in biomedical field, where the collagen products are intended to have intimate contact with human tissues or be administered parenterally.

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