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Thermoresponsive elastin-*b*-collagen-like peptide bioconjugate nanovesicles for targeted drug delivery to collagen-containing matrices

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Abstract

Over the past few decades, (poly)peptide block copolymers have been widely employed in generating well-defined nanostructures as vehicles for targeted drug delivery applications. We previously reported the assembly of thermoresponsive nanoscale vesicles from an elastin-*b*-collagen like peptide (ELP-CLP). The vesicles were observed to dissociate at *elevated* temperatures, despite the LCST-like behavior of the tethered ELP domain, which is suggested to be triggered by the unfolding of the CLP domain. Here, the potential of using the vesicles as drug delivery vehicles for targeting collagen-containing matrices is evaluated. The sustained release of an encapsulated model drug was achieved over a period of three weeks, following which complete release could be triggered via heating. The ELP-CLP vesicles show strong retention on a collagen substrate, presumably through collagen triple helix interactions. Cell viability and proliferation studies using fibroblasts and chondrocytes suggest that the vesicles are highly cytocompatible. Additionally, essentially no activation of a macrophage-like cell line is observed, suggesting that the vesicles do not initiate an inflammatory response. Endowed with thermally controlled delivery, the ability to bind collagen, and excellent cytocompatibility, these ELP-CLP nanovesicles are suggested to have significant potential in the controlled delivery of drugs to collagen-containing matrices and tissues.

TOC image

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Supporting information

Drug release schematic, collagen retention schematic, ImageJ analysis, drug release first order fit, dynamic light scattering of fluorescein labelled ELP-CLP vesicles, metabolic activity of RAW264.7 macrophages, fibroblast viability, ATDC5 viability and cell area coverage. This material is available free of charge at <http://pubs.acs.org>.

Notes:

The authors declare no conflict of interest.

Keywords

Collagen-like peptide; elastin-like peptide; self-assembly; thermoresponsive; drug delivery; cytocompatibility

1. Introduction

Collagen is the main component of the extracellular matrix (ECM) in humans. Largely found in tendon, ligament, skin, cartilage and bone, collagens have widespread functions not only as a structural element in tissues, but also in mediating cell adhesion, migration, tissue scaffolding and repair.¹ Although the roles of the 29 identified collagens *in vivo* vary widely,² they all share the same tertiary structure – collagen triple helix, which comprises three polyproline-II type helices twisted together in a right-handed form.³ Each strand of the helix consists of regularly repeated Gly-X-Y tripeptide motifs, with proline (Pro) and (4R)-hydroxyproline (Hyp) occupying the X and Y positions at the highest frequency. Short synthetic collagen-like peptides (CLPs) (or collagen-mimetic peptides (CMPs)) mimic the triple helical conformation of native collagens,^{4–6} although unlike native full-length collagens, the folding and unfolding of CLP triple helices are reversible as a result of their relatively low molecular mass (<5 kDa).⁶ Over the past few decades, these peptides have been widely employed to elucidate the triple helix structure and the stabilization effects of different amino acid residues in collagens,^{7–11} as well as to produce higher-order supramolecular structures.^{12–16} More recently, the strong propensity of single-stranded CLPs to bind native collagen via a strand invasion process has been reported¹⁷ and has permitted the utilization of CLPs as staining reagents to detect minute quantities of collagens in human tissues including skin, cornea, bone¹⁸ and liver.¹⁹ *In vivo* studies suggest that CLPs are able to permeate and accumulate in tissues with high levels of matrix metalloproteinase (MMP) activities and/or collagen damage, such as prostate tumor xenografts, tendon,²⁰ and articular cartilage.²¹ Despite the great potential of using CLPs as a novel biomaterial for tracking pathological collagens, the utilization of these peptides as building blocks in generating nanoscale drug carriers is very limited.^{22–25}

Derived from tropoelastin,²⁶ which is the other major structural protein in mammals, elastin-like polypeptides (ELPs) comprise multiple copies of the consensus repeat of the native protein, the pentapeptide Val-Pro-Gly-Xaa-Gly (VPGXG).^{27–31} This sequence is widely known for its LCST-like inverse temperature transition, and ELPs have been very widely used in the development of drug delivery vehicles that can be targeted to tissues and cells either via passive or peptide- and stimuli-responsive mechanisms.^{32–38} However, essentially all the ELPs employed to date have been recombinantly synthesized, with tens or even hundreds of pentapeptide repeats. Thermoresponsive drug delivery systems comprising short synthetic ELPs (fewer than ten pentapeptides) have not been used for such applications due to their high transition temperatures,^{39, 40} although limited reports exist of the thermoresponsive behavior of synthetic polymers equipped with large numbers of short ELPs.^{41–43}

In our previous work,⁴⁴ the high transition temperature of short ELPs was reduced to experimentally viable temperatures by the conjugation of the short ELPs with a triple-helix-forming collagen-like peptide with the sequence (GPO)₄GFOGER(GPO)₄GG. The CLP exhibited a melting temperature at 50 °C, which enables formation of stable triple helix at physiological temperature. The peptide sequence GFOGER was employed owing to the fact that it is widely recognized by several kinds of integrins such as $\alpha_1\beta_2$, $\alpha_2\beta_1$, and $\alpha_{11}\beta_1$.^{45–47} The ELP consisted of 6 repeats of the VPGFG pentapeptide. While the ELP itself showed an inverse transition temperature (T_i) above 80 °C, the resulting ELP-CLP conjugate exhibited a remarkable reduction in T_i of the ELP domain to below 4 °C, attributed to the formation of the CLP triple helix, which results in a locally high concentration of the ELP domains. The lower transition temperature of the conjugate enabled the facile formation of well-defined nanovesicles with a diameter of approximately 160 nm at physiological temperature and the unexpected resolubilization of the vesicles at *elevated* temperatures upon unfolding of the CLP domain. The LCST-like transition of the ELP domain, coupled with the dissociation of the vesicles at elevated temperatures, should provide these vesicles with significant potential in thermally controlled drug release. In this work, fluorescein, as a model hydrophobic cargo, was encapsulated into the vesicles; the sustained release of the cargo was measured over a period of 3 weeks, as well as the potential for thermally-triggered burst release.

Previous studies conducted by Yu and coworkers suggested that unfolded, single-strand collagen-like peptides had a strong propensity to target native collagens via highly efficient and specific triple helix hybridization.^{5, 17–19, 21, 48–53} Based on these reports, we speculated that the interactions between CLPs on the surface of the ELP-CLP vesicles and native collagens¹⁷ could be exploited to target these vesicles to collagen-containing substrates. In this work, this hypothesis was corroborated by retention experiments of the vesicles on type II collagen films. The cytocompatibility of the ELP-CLP vesicles was evaluated by measuring cell viability *in vitro*, using NIH-3T3 fibroblasts, ATDC5 chondrocytes, as well as RAW 264.7 macrophages, to evaluate their potential for future *in vivo* studies. This work suggests that the ELP-CLP nanovesicles could be promising candidates as collagen-targeting drug carriers, especially for diseases associated with high collagen remodeling such as osteoarthritis,^{54–57} rheumatoid arthritis,^{58–60} Marfan syndrome,^{21, 61} cancer,^{62–64} and osteogenesis imperfecta.⁶⁵

2. Materials and methods

2.1 Materials

Fmoc-protected amino acids including Fmoc-propargyl glycine, Rink amide MBHA resin, N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU), and piperidine for solid-phase peptide synthesis were purchased from AAPPTec Inc. (Louisville, KY). HPLC-grade acetonitrile, dimethylformamide (DMF), copper wire, acetic acid, Dulbecco's phosphate buffered saline (PBS), 8-well plates for collagen retention experiments, calcein AM, propidium iodide, Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12), Opti-MEM I reduced serum media and Hank's balanced salt solution (HBSS) were purchased from Thermo Fisher Scientific (Fairlawn, NJ). Copper(I) acetate, 4-azidobutanoic acid, N-methyl-2-pyrrolidone (NMP), trifluoroacetic acid (TFA), triisopropylsilane (TIS), triethylamine (TEA), diisopropylethylamine (DIEA), deuterated DMSO, collagen from chicken sternal (Type II), lipopolysaccharide from E. coli O55:B5 (LPS), and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum, penicillin streptomycin, and 12-well tissue culture plates were purchased from Corning Mediatech Inc. (Manassas, VA). NIH-3T3 embryonic murine fibroblasts and RAW264.7 murine macrophages were purchased from American Type Culture Collection (Manassas, VA). ATDC5 cells were kindly provided by the laboratories of Dr. Randall Duncan at the University of Delaware. β -mercaptoethanol, antibiotic/antimycotic, and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) were purchased from GIBCO (Gaithersburg, MD). PrestoBlue Viability Assay was obtained from Life Technologies (Carlsbad, CA). TNF- α ELISA was obtained from BD Bioscience (San Jose, CA). Ethidium Homodimer-1 (EthD-1) was purchased from Applied BioProbes (Rockville, MD).

2.2 ELP-CLP diblock synthesis and characterization

As described in our previous paper,⁴⁴ an N-terminal azide-functionalized CLP with the sequence (GPO)₄GFOGER(GPO)₄GG and a C-terminal alkyne-functionalized elastin-like peptide with sequence (VPGFG)₆G' (G': propargyl glycine) were synthesized via traditional solid-phase peptide synthesis methods (SPPS) using an Focus XC automatic peptide synthesizer (AAPPTec Inc., Louisville, KY). The CLP was functionalized with 4-azidobutanoic acid on-resin. Crude peptides were purified via reverse-phase HPLC (Waters Inc., Milford, MA) on a Waters Xbridge BEH130 Prep C-18 column. The molecular weights of the peptides were confirmed via electrospray ionization mass spectrometry (ESI-MS, AutospecQ, VG Analytical, Manchester, UK) and the purity of the peptide was confirmed via analytical scale, reverse-phase HPLC (Waters 2996; Symmetry C18, 3.5 μ m, 4.6 $\times$ 75 mm).

The synthesis of the ELP-CLP conjugate was achieved via copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) "click" reaction (Scheme 1). The reaction was carried out in DMF at 80 °C for 24 hours, using a 1:1 ELP: CLP molar ratio, with the addition of 0.25 equivalent copper(I) acetate and copper wire. After reaction, the resulting hybrid copolymer was isolated into cold diethyl ether. The diblock was then redissolved in water and dialyzed against water at room temperature for 7 days to remove the residual catalyst. Water was

changed twice on day 1 and once daily from day 2 to day 7. The purity of the diblock was verified via gel permeation chromatography (GPC), ^1H nuclear magnetic resonance spectrometry (NMR) and Fourier transform infrared spectroscopy (FTIR). The ability of the CLP domain to retain its triple helical conformation, as well as its melting temperature, were studied using circular dichroism spectroscopy (Jasco 810 circular dichroism spectropolarimeter, Jasco Inc., Easton, MD, USA). Details on the characterization of the ELP-CLP diblock have been previously reported.⁴⁴

2.3 Thermoresponsive cargo release from ELP-CLP nanovesicles

2.3.1 Preparation of cargo-loaded vesicles—To investigate the feasibility of the ELP-CLP nanovesicles as potential cargo carriers, fluorescein was used as a model cargo and encapsulated into the vesicles. Briefly, 0.5 mg ELP-CLP diblock conjugate and 1 mg fluorescein were dissolved in 0.5 mL PBS (10 mM, pH 7.4) at 80 °C. The sample was then incubated at 37 °C for 1 hour to allow vesicle formation and simultaneous encapsulation of fluorescein into the vesicles. Vesicle solutions were then concentrated by centrifugation at 37 °C, using an Amicon Ultra-0.5 mL Centrifugal Filters (MWCO 3 kDa, Billerica, MA) and were washed at least 10 times with 0.5 mL fresh PBS (pre-incubated at 37 °C) until no fluorescence was detected in the wash solution. Concentrated vesicle solutions were then diluted using fresh PBS to yield 1 mg/mL working solutions of vesicles for subsequent studies. For transmission electron microscopy (TEM) studies, instead of using PBS, vesicles were prepared in deionized water to prevent the precipitation of salts and phosphotungstic acid (PTA) stain.

2.3.1 Size distribution of vesicles—Analysis of vesicle sizes prior to and after fluorescein encapsulation was conducted via dynamic light scattering (DLS) on a ZetaSizer Nano Series (Nano ZS, Malvern Instruments, U.K.) at a scattering angle of 173°. A He-Ne laser at 633 nm wavelength was used at an intensity of 4 mW. Data was fitted using the cumulant method. Size distribution studies were performed at both 37 °C and 80 °C. Each sample was incubated for 10 min at the desired temperature before measurements.

2.3.3 Morphology—The morphology of the vesicles, prior to and after fluorescein encapsulation, was investigated via transmission electron microscopy (TEM). Samples were prepared on carbon-coated copper grids (CF300-Cu, Electron Microscopy Sciences Inc.). The grids, pipette tips, and samples were all incubated in an isothermal oven (VWR Signature™ Forced Air Safety Ovens, VWR Inc.) at desired temperatures (37 °C or 80 °C) for at least 30 min before sample preparation, which was also conducted in the oven. 5 μL of the sample solution was drop cast on the grid and blotted after 60 seconds. For staining, 1% phosphotungstic acid (PTA) (pH adjusted to 7.0 using 1 M NaOH) as a negative stain was used. 3 μL of the PTA solution was drop cast on the grid and blotted after 10 seconds. The sample was then allowed to dry in the oven at the desired temperature for 30 minutes and then was air-dried for 2 hours. TEM images were taken on a JEM-3010 TEM (JEOL USA Inc., Peabody, MA) at an acceleration voltage of 300 keV. Size distribution and wall thickness of the vesicles from TEM were analyzed using ImageJ. A total number of 233 vesicles from 8 images were analyzed for the fluorescein-free samples and a total number of 147 vesicles from 8 images were analyzed for the fluorescein-loaded samples.

2.3.4 Release of fluorescein from vesicles—The release profile of encapsulated fluorescein from the ELP-CLP vesicles was investigated at physiological temperature. A 0.5 mL sample of fluorescein-loaded vesicles in solution at 1 mg/mL in PBS (10 mM, pH 7.4) was dialyzed against 14 mL PBS using a 0.5 mL Slide-A-Lyzer™ MINI Dialysis Device (MWCO 3.5 kDa, Thermo Fisher Scientific Inc, Waltham, MA) at 37 °C. At predetermined time points, 3 mL samples were collected and replenished with fresh PBS. The release of fluorescein was monitored by measuring the fluorescence intensity of the collected sample, using a PerkinElmer Fusion microplate reader (Waltham, MA, USA), with excitation/emission wavelengths of 485/535 nm. The measurements were performed at room temperature. At day 21, the vesicle solution was mixed with the PBS dialysis solution and incubated at 80 °C for one hour to fully dissociate the vesicles and the amount of previously unreleased fluorescein that was liberated upon vesicle dissolution was measured. The cumulative amount of total released fluorescein after heating was used as the amount of encapsulated fluorescein. The reported data represent an average of three individual experiments. A detailed schematic of the experiment is shown in Figure S1.

2.4 Vesicle retention on collagen films

Equipped with CLP domains on the exterior layer of the ELP-CLP vesicles, these vesicles were expected to bind with native collagens via triple helix hybridization. Thus, particle retention experiments were performed on collagen films. Type II collagen from chicken sternal cartilage was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved at 5 mg/mL in 0.25% v/v acetic acid overnight. The pH of the collagen solution was adjusted to 7-8 using 10x PBS and 1 M NaOH. To avoid complications with the potential release of free fluorescein from immobilized vesicles, carboxyfluorescein was covalently conjugated to the N-terminal amine of the ELP. The fluorescein-labelled ELP-CLP vesicles (FELP-CLP, 210 µg/mL in PBS, pH 7.4) were incubated at 65 °C for 15 minutes to partially unfold the CLP triple helices.

In previous experiments, the specific binding of CLP-modified nanoparticles to collagen substrates, versus their simple physical entrapment, was demonstrated for nanoparticle binding to dried collagen I films that were rehydrated in the presence of CLP-modified DNA polyplexes.⁶⁶ In the studies reported here, however, the collagen II substrate was partially dissolved upon the preincubation of the film with heated ELP-CLP vesicle solutions. Thus, a non-preincubated vesicle solution was employed as a negative control to separate the effect of physical entrapment versus specific binding via triple helix hybridization. For each sample, 30 µL of vesicle solution was added to 600 µL of 4 °C preincubated collagen solution to obtain a final vesicle concentration of 10 µg/mL. The vesicle/collagen samples were then incubated at 4 °C for 3 hours to allow the vesicles to bind with the collagen via triple helix hybridization; the vesicle-containing collagen solutions were then gelled at 37 °C for 1 hour and dried overnight at room temperature to form a thin collagen film that remained intact throughout the extended incubation times of the release experiment. The films were then washed with fresh PBS twelve times until no fluorescence was detected in the wash, to ensure all unembedded vesicles were removed. A detailed schematic of the experiment can be found in Figure S2.

To study the release of FELP-CLP vesicles from the films, 500 μL 1x PBS was added on the top of each film as a reservoir. A 200 μL sample was collected and 200 μL fresh PBS was added to each well at desired timepoints (12 hour, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days and 10 days). The mass of vesicles released was calculated by measuring the fluorescence intensity, using a PerkinElmer Fusion microplate reader (Waltham, MA, USA), at excitation/emission wavelengths of 485/535 nm. Preformed FELP-CLP vesicle solutions at various concentrations were prepared as calibration standards. Results are reported as an average of 4 repeats for the preheated samples and an average of 3 repeats for the non-preheated controls. At day 10, the films were heated to 80 $^{\circ}\text{C}$ to fully dissolve the film and the amount of remaining vesicles liberated from the films was measured with fluorescence measurements.

2.5 Macrophage activation

RAW264.7 murine macrophages were cultured in DMEM supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 55 μM β -mercaptoethanol, and 1% antibiotic/antimycotic at 37 $^{\circ}\text{C}$ with 5% CO_2 . Upon reaching 60-80% confluency, cells were lifted off the plates by gentle scraping and seeded in a 96-well plate at a density of 150,000 cells/ cm^2 and 250,000 cells/mL in cell culture medium. After an overnight incubation and media change, cells were treated with various concentrations of ELP-CLP vesicles (50, 150, 500, and 1000 $\mu\text{g}/\text{mL}$) in medium. Cells treated with LPS (50 ng/mL) served as a positive control, while cells in culture medium without the vesicles were utilized as a negative control. Cell metabolism post-treatment was assayed, according to the manufacturer's instructions, using the PrestoBlue viability assay, which provides a fluorescence measurement of the overall reductive capability of the cells. Briefly, 20 μL of reagent was added to 100 μL of cells and medium. After 2 h of incubation at 37 $^{\circ}\text{C}$, the medium was collected and analyzed using a PerkinElmer VICTOR3 1420 Multilabel Plate Reader (PerkinElmer, Waltham, MA) at excitation/emission wavelengths of 570 nm/615 nm. After 8 h of incubation, the supernatant was collected and stored at -20°C until further analysis. Tumor necrosis factor- α (TNF- α) concentration in collected media samples was measured via an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions.

2.6 Fibroblast viability

The cytotoxicity of the ELP-CLP vesicles was assessed *in vitro* for the murine embryonic fibroblast (NIH-3T3) cell line. Cells were cultured for 24 hours in a 12-well Corning Cellgro $^{\circ}$ tissue culture plate (plating density of 10,000 cells/ cm^2 , working volume of 1 mL) in Dulbecco's Modified Eagle's Media (DMEM) (supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin streptomycin (PS)), and then were washed with 1 mL of Dulbecco's phosphate buffered saline (DPBS) two times. The cells were then incubated in fully supplemented DMEM media for 24 hours, in the presence of preformed ELP-CLP vesicles at final concentrations of 0, 500, and 1000 $\mu\text{g}/\text{mL}$. After the 24-hour incubation, the cells were then washed again with DPBS, stained with non-supplemented DMEM containing a final concentration of 2.5 $\mu\text{g}/\text{mL}$ calcein AM ($\lambda_{\text{ex}} \approx 495$ nm) and 1.0 $\mu\text{g}/\text{mL}$ propidium iodide ($\lambda_{\text{ex}} \approx 535$ nm) for 45 minutes, and were imaged using a Leica DMI-6000B fluorescence microscope at 4 $\times$ magnification. A positive-toxic control of dead

cells was used to verify the detection of propidium iodide staining of dead cells (data not shown).

2.7 Chondrocyte viability and proliferation

ATDC5 cells, a murine chondrocyte cell line, were plated in 12-well dishes (density of 10,000 cells/cm²) and cultured for 24 hours in the presence of DMEM/F-12 (1:1 mixture) containing 5% v/v FBS and 1% v/v PS (working volume of 1 mL/well). Cells were then washed once with 1x HBSS and preformed ELP-CLP vesicles (in 1x PBS) were added to the wells at 0, 50, 150, 500, and 1000 µg/mL. Following 24 or 72 hours of incubation with the ELP-CLP vesicles, the culture medium was removed, cells washed with 1x HBSS, and calcein-AM (2 µM; $\lambda_{\text{ex}} \approx 495$ nm) and EtDH-1 (4 µM; $\lambda_{\text{ex}} \approx 535$ nm) in Opti-MEM media were added for 40 minutes prior to imaging using an Axio Observer Z1 fluorescence microscope (10× magnification). To quantitate cell viability, the number of live and dead ATDC5 cells were counted manually. Cell proliferation was measured longitudinally (0-through 72 hours) via manual counting, as well as measures of daily change in areal cell coverage (area covered by cells/total area of image) from phase contrast images (10× magnification) using a custom MATLAB (Version R2015a, The MathWorks, Natick, MA) object detection method, similarly to previously reported methods.⁶⁷

2.8 Statistical analysis

Results are expressed as the mean $\pm$ standard error of the mean, unless otherwise noted. Statistical comparisons for the RAW264.7 macrophage TNF- α production were calculated using a one-way analysis of variance (ANOVA). Statistical comparisons for the ADTC5 cell live/dead percentage and cell number were calculated using a two-way analysis of variance (ANOVA). A p-value of <0.05 was considered significant. Pairwise comparisons were made using a Tukey HSD post-hoc analysis. Statistical interpretations were made via use of JMP Pro 12 (SAS Institute Incorporated).

3. Results

3.1 Thermoresponsive cargo release from ELP-CLP vesicles

Nanostructures self-assembled from amphiphilic block copolymers, including those comprising one thermoresponsive block, have been intensely investigated and widely employed as drug delivery vehicles over multiple decades.^{68–73} In addition to traditional synthetic thermoresponsive polymers such as poly(N-isopropylacrylamide) (PNIPAAm),^{74–78} (poly)peptides with desired thermoresponsiveness such as elastin-like polypeptides (ELPs) have also been reported.^{79–83} The characteristic, easily tunable, LCST-like inverse temperature transition of this class of polypeptides^{79, 84} provides ELPs with the ability to be used as building blocks for thermoresponsive smart biomaterials.^{85, 86} In addition to the production of ELP block copolypeptides,^{37, 87–100} ELP peptides have also been conjugated to synthetic polymers to generate thermoresponsive assemblies.^{34, 101, 102}

However, while ELPs have been widely utilized to form bioconjugates with desired thermoresponsiveness, essentially all the ELPs employed have been recombinant. There have been very limited reports of thermoresponsive nanostructures that can be assembled

from short synthetic ELPs,^{41–43} likely due to their high transition temperatures.^{39, 40} As mentioned above, we have demonstrated that this limitation can be overcome by conjugating short ELPs with a triple-helix-forming collagen-like peptide (CLP). The resulting ELP-CLP diblock self-assembled into well-defined vesicles at physiological temperature. More importantly, when the diblock assemblies were heated above the melting temperature (T_m) of the CLP domain, the vesicles became fully solubilized, likely attributed to the unfolding of the CLP triple helices. This thermoresponsive dissociation may endow the vesicles with potential for temperature controlled drug release. In this work, fluorescein, as one of the most widely used fluorescent probes,^{103–108} was encapsulated into the ELP-CLP vesicles as a model drug to test the capacity of the ELP-CLP vesicles for thermally triggered cargo release. With an octanol/water partition coefficient value (logP) of 3.4, which is similar to those measured for a wide range of drugs for the treatment of rheumatoid arthritis (e.g. hydroxychloroquine, leflunomide, sulfasalazine), osteoarthritis (e.g. duloxetine) and cancer (e.g. paclitaxel, imatinib),^{109–112} fluorescein serves as a good candidate as a fluorescent model drug.

3.1.1 Vesicle characterization—Dynamic light scattering (DLS) was conducted to study the size distribution of the ELP-CLP vesicles, with or without fluorescein encapsulated, in order to ascertain any potential effect of encapsulation on vesicle morphology. Figure 1a and Figure 1b show the size distributions for the diblock sample at 37 °C and 80 °C, with or without fluorescein encapsulation, respectively. In both cases, nanostructures with an average diameter of approximately 100 – 200 nm were observed, consistent with our previous studies.⁴⁴ As expected, when the sample was heated to 80 °C, the nanostructures dissociated into small objects with diameters of approximately 5 – 10 nm, likely attributable to the resolubilization of the diblock monomer caused by unfolding of the CLP triple helix.

It is noteworthy that the thermoresponsive behavior of the ELP-CLP conjugates is very different from traditional thermoresponsive synthetic polymers, and is also different from thermoresponsive polymer-CLP conjugates we have previously reported.^{23, 25} The ELP-CLP diblock is a dual thermoresponsive system, with the thermally responsive behavior of the ELP domain enabled by the anchoring effect of the conjugated CLP triple helix; the ELP shows no thermoresponsive behavior when in its monomeric form. Thus, when the CLP triple helix unfolds, the LCST of the ELP domain increases drastically, which leads to the dissociation of the vesicles.

In a previous study from our group,²⁵ the thermoresponsive behavior of CLP conjugated with a thermoresponsive synthetic polymer (poly(diethylene glycol methyl ether methacrylate) (PDEGMEMA)) was evaluated. Like the ELP-CLP diblock system discussed in this report, the PDEGMEMA-CLP diblock formed nanoparticles above the LCST of the polymer domain. However, unfolding of the CLP triple helices above the T_m of the CLP domain did not trigger the dissociation of the PDEGMEMA-CLP nanoparticles, owing to the fact that the PDEGMEMA exhibits an LCST transition even when not conjugated to the CLP. Thus, the short ELP domains employed in this work are required for the triggered dissociation of the ELP-CLP vesicles at elevated temperatures.

To obtain insight into the morphologies of the nanostructures, transmission electron microscopy (TEM) measurements were conducted on the fluorescein-free (Figure 2a and 2b) and fluorescein-loaded vesicles (Figure 2c and 2d) at 37 °C and 80 °C; visualization was facilitated via use of 1% PTA aqueous solution (pH adjusted to 7.0) as a negative stain. At physiological temperature, both samples self-assembled into nanoscale vesicles (Figure 2a and 2c). Size distribution analysis via ImageJ suggested the measured diameters of the vesicles (186 ± 51 and 138 ± 34 nm for the cargo-free and cargo-loaded vesicles, respectively) were highly consistent with DLS results (Figure S3). Interestingly, a more distinct vesicular structure was observed for the fluorescein-loaded vesicles, which may be attributable to an increase in hydrophobic interactions in the collapsed ELP domain with the addition of encapsulated fluorescein. Another possible reason could be that the PTA stain was capable of diffusing into and accumulating at both the exterior and interior surfaces of the vesicles, as we have previously reported.⁴⁴ This hypothesis was also corroborated by the slight reduction of the diameter of the vesicles when loaded with fluorescein (black curves in Figure 1a and 1b). ImageJ analysis suggests the thickness of the vesicle bilayer to be approximately 21.8 ± 2.9 nm, similar to the bilayer thickness observed for fluorescein-free ELP-CLP vesicles.⁴⁴ Similar vesicular structures and sizes have also been reported in previous model drug carrier systems, including polymeric vesicles for the delivery of doxorubicin^{113–117} and paclitaxel,^{118, 119} as well as thermoresponsive recombinant ELP-based vesicles.^{34, 102} As expected, no well-defined structures were observed at 80 °C (Figure 2b and 2d), indicative of full dissociation of the vesicles above the T_m of the CLP domain. The results confirmed that the vesicle-forming ability of the ELP-CLP diblock at physiological temperature was retained with the encapsulation of the hydrophobic cargo, and that these structures are competent for dissolution at elevated temperatures.

3.1.2 Release profile—The release of encapsulated fluorescein from the vesicles at physiological temperature (37 °C) was monitored as a function of time by measuring the fluorescence intensity of the buffer against which the vesicles were dialyzed. The data were normalized to the total amount of encapsulated fluorescein and cumulative release within a period of 3 weeks was plotted in Figure 3. At day 21, the sample was heated to 80 °C to dissociate the vesicles. As shown in Figure 3, a rapid release of the remaining 45% of the initially encapsulated cargo was observed after heating. Modelling of the initial 8-hour portion of the release data according to first-order kinetics yields a release rate constant of 0.056 h^{-1} (Figure S4) with excellent fit ($R^2 = 0.998$). We hypothesize that the observed rapid release within the first day may be attributed to the release of non-specifically bound fluorescein on the surface of the vesicles. A subsequent slow release of approximately 5% additional fluorescein was observed from day 2 to day 21 (Figure 3), indicating the remaining encapsulated cargo was well retained in the vesicles.

Similar release profiles consisting of a rapid release in the early stage and a subsequent slow release has also been reported in several types of drug carriers such as poly-ε-caprolactone (PCL) microspheres,¹²⁰ poly(lactic-co-glycolic) acids (PLGAs) nanoparticles,¹²¹ and polymeric micelles consisting of hydrophobic alkyl chain core and PEG corona,¹²² for the delivery of ibuprofen,¹²⁰ ketoprofen, indomechacin,¹²¹ and lidocaine.¹²²

Importantly, at day 21, when the sample was heated up to unfold the CLP domain, a burst release of all the remaining encapsulated fluorescein was observed, almost certainly triggered by the dissociation of the vesicles at elevated temperatures, as discussed in the previous section (Figure 1b and 2d). When the CLP triple helix is unfolded at 80 °C, the anchoring of multiple ELP domains is lost, and the ELP-CLP diblock monomer becomes soluble, so it is unlikely that the fluorescein is associated with the peptide. The total amount of released cargo was 0.18 µg, which exceeds, by approximately 1-10-fold, the IC₅₀ values reported for a range of hydrophobic drugs for the treatment of osteoarthritis,^{123–125} rheumatoid arthritis,¹²⁶ and cancer,^{127, 128} suggesting that these ELP-CLP vesicles may have potential to serve as a drug carrier for such applications. In order to test the longer-term efficacy of the delivery system, we have also preliminarily studied the release profile over 42 days. A sustained slow release over a period of 6 weeks was observed, followed by a temperature-triggered burst release at day 42 (Figure S5).

Although the temperature at which release is triggered in the studies reported here is not physiologically relevant, these results nevertheless successfully demonstrate that the encapsulation/release of hydrophobic cargo can be triggered by the dissociation of the vesicles upon unfolding of the CLP triple helix. The transition temperature, and likely rate of release, could therefore be tuned by changing the T_m of the CLP triple helix; investigations on the manipulation of this transition temperature are underway.

Because both the ELP and CLP are very short and contain no enzyme-specific (matrix metalloproteinase, elastase, or collagenase) degradable motifs, enzymatic digestion of the diblock conjugate in future *in vivo* application is not expected. Indeed, the stability of CLPs *in vivo* was previously studied by Yu and coworkers,²¹ with results suggesting that CLPs were stable in PC-3 prostate tumors for at least 4 days. Future investigations of these ELP-CLP vesicles *in vivo*, currently underway in our laboratories, will include assessments of their stability.

3.2 Collagen retention

Equipped with collagen domains on the exterior surface, these ELP-CLP vesicles should be competent for targeting native collagens via triple helix hybridization,^{5, 17} for the treatment of diseases with high collagen remodeling activities, such as osteoarthritis,^{56, 57} rheumatoid arthritis,^{58–60} and cancer.^{62–64} To make an initial determination of the immobilization of ELP-CLP diblock vesicles on collagen-containing matrices, vesicle binding and retention studies were conducted on films made from type II collagen, which is the principle type of collagen (85 – 90 %) in the articular cartilage matrix.¹²⁹ Since the retention and release of vesicles is most easily quantified via fluorescence measurements and the vesicles demonstrated the release of encapsulated fluorescein (Figure 3), in order to distinguish the two different kinds of release, the ELP-CLP diblock was covalently functionalized with fluorescein at the N-terminal end (FELP-CLP) instead of via physical encapsulation. DLS results confirmed that the fluorescein-labelled diblock was still able to self-assemble into vesicles with similar size as the unmodified ELP-CLP diblock (Figure S6).

To increase the likelihood of the vesicles binding to the collagen substrate via triple helix hybridization, the fluorescein-labelled vesicles were pre-incubated at 65 °C for 15 minutes,

prior to their addition to the collagen films, to allow the CLP triple helix domain to be partially unfolded. DLS experiments indicated that the ELP-CLP diblock was able to maintain its assembled state under such conditions (Figure S6). A non-preincubated sample was used as a negative control. The release profiles of the preincubated sample vs. unpreincubated control are shown in Figure 4. After 10 days, only $0.071 \mu\text{g}/\text{cm}^2$ (11.2%) of the vesicles were released from the film, illustrating that vesicles were strongly retained in the collagen film regardless of the pre-incubation treatment. At day 10, the samples were heated at 80°C and the collagen films were dissolved, resulting in complete dissolution and release of the vesicles into solution, and allowing estimation of the original amount of vesicles sequestered in the film at the start of the experiment. As shown in Figure 4, while a total of approximately $0.20 \mu\text{g}/\text{cm}^2$ vesicles from the negative control were detected (and thus initially retained on the film), the collagen films treated with preincubated vesicles showed increased sequestration, with $0.63 \mu\text{g}/\text{cm}^2$ vesicles detected, approximately 3-fold higher than the negative control. The improved retention for the preincubated sample is almost certainly attributable to the specific binding of the vesicles via triple helix hybridization versus the physical encapsulation of the non-incubated vesicles that resulted from the bulk drying process (which was necessary to make robust collagen films). These results indicated that the ability of the CLP to target collagen matrices via triple helix formation was well retained in ELP-CLP vesicles assembled after conjugation of the CLP with the ELP domain.

It is noteworthy that the high T_m of the CLP employed in the current study necessitated an initial pre-incubation to achieve efficient incorporation into collagen films. While the high T_m of the CLP may not be particularly relevant for future temperature-triggered release in a clinical application, the requirement for preincubation supports the fact that the incorporation of the ELP-CLP vesicles into collagen is in fact mediated by triple helix strand exchange of the CLP in the vesicles with the collagen fibrils in the film. Such strong binding and high retention on collagen substrates have also been reported previously for other CLP-modified nanoparticles. For example, gold nanoparticles (Au-NP) conjugated with short CLPs showed specific binding with reconstituted type I collagen fiber, while Au-NP functionalized with a scrambled G7P7O7 peptide, incapable for forming triple helix, exhibited little binding with the collagen fibers.⁴⁸ By conjugating a CLP-polymer hybrid as an amphiphile to stabilize a conducting polymer (poly(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-(2,10,3)-thiadiazole)) (PFBT), the resulting nanoparticles showed strong binding affinity with collagen substrate and were used as histological staining reagent for mouse corneal tissue sections, while the PEG-conducting polymer nanoparticle control exhibited negligible staining.⁵⁰ More recently, the Kiick and Sullivan laboratories have employed CLPs to control the retention and delivery of DNA polyplexes from collagen matrices, including both 2-D collagen films and 3-D hydrogels.⁶⁶ The results indicate substantially improved retention and gene expression of the CLP-modified polyplexes compared with unmodified polyplexes.

Based on the high retention of hydrophobic compounds in the vesicles and their subsequent temperature-controlled release, coupled with the high retention of the vesicles in collagen films, the ELP-CLP vesicles show potential to be used as carriers of hydrophobic drugs, with temperature-controlled drug release profiles, as well as the ability to target pathological

collagens. To evaluate the potential of these vesicles toward these types of applications, the inflammatory response and cytocompatibility of the vesicles were explored.

3.3 Cytocompatibility of the ELP-CLP vesicles

One application where ELP-CLP vesicles hold promise as a candidate collagen-targeting drug carrier is in the treatment of arthritic and rheumatic diseases. Drug therapy within the intra-articular space of joints, such as the knee, is challenging due to the lack of tissue-specific targeting of cartilage and the rapid capillary and lymphatic clearance of therapeutic compounds once introduced into the joint space.¹³⁰ The collagen targeting, retention, and drug-release properties of ELP-CLP vesicles may thus suggest their potential as drug carriers for the localized treatment of joint disease. The initiation and progression of joints disorders involves the activation or dysregulation of a variety of cell types, including chondrocytes, fibroblast-like synoviocytes, macrophage-like synoviocytes, and inflammatory cells.^{131–134} Thus, to evaluate the cytocompatibility of the ELP-CLP vesicles, we evaluated the effect of ELP-CLP vesicles on macrophage, fibroblast, and chondrocyte-like cell lines *in vitro*.

3.3.1 Macrophage activation—The use of biomaterials within tissues can evoke inflammatory reactions, mediated by multiple cell types and signaling molecules, and cause abnormal wound healing, and potentially damage host tissue.¹³⁵ In particular, macrophages play a critical role in the inflammation process by secreting pro-inflammatory cytokines that are heavily involved in tissue repair and foreign body reactions.¹³⁶ Indeed, the activation of macrophage-like (Type A) synoviocytes is critically implicated in the pathology and progression of both osteoarthritis and rheumatoid arthritis.^{131, 134} Therefore, to gauge the potential suitability of ELP-CLP vesicles for *in vivo* applications, including joint delivery, we evaluated the inflammatory potential of our materials against murine-derived RAW264.7 macrophage-like cells. TNF- α is a pro-inflammatory agent that is rapidly released by activated macrophage and plays an important role in regulating the pro-inflammatory cytokine production, inflammatory cell recruitment, and extracellular matrix catabolism.^{137, 138} Owing to the role of TNF- α as an early mediator of inflammation, we evaluated TNF- α production from RAW264.7 cultures after 8 hrs of incubation with the ELP-CLP vesicles. As shown definitively in the data, RAW264.7 macrophages produced negligible amounts of TNF- α following culture with 50, 150, 500, or 1000 $\mu\text{g/mL}$ of ELP-CLP vesicles, similar to the low activation level displayed by control macrophages exposed to medium only and nearly 100-fold lower than RAW264.7 cells exposed to 50 ng/mL LPS as a positive control (Figure 5). Macrophage metabolic activity was similar across all cultures (Figure S7), indicating that the ELP-CLP vesicles were not toxic at any of the concentrations tested suggesting their cytocompatibility. Thus, the non-inflammatory character of the ELP-CLP vesicles is suggested by the minute levels of TNF- α expression observed in macrophage-like cultures following vesicle treatment. This result is not unexpected as excellent biocompatibility has been reported for other ELP-based biomaterials both *in vitro* and *in vivo*.^{139, 140} Further, the low levels of TNF- α expression observed here are consistent with the responses observed *in vitro* with poly(N-isopropylacrylamide)¹⁴¹ and poly(lactic-co-glycolic acid)¹⁴² nanoparticle systems, where macrophage production of TNF- α was unaltered following vesicle treatment. As such, the lack of significant activation of

macrophages by the ELP-CLP vesicles suggests their potential as a biocompatible drug delivery system.

3.3.2 Fibroblast viability—Considering the critical role of fibroblasts in the synthesis of collagen matrices in the ECM, as well as in the progression of arthritic diseases,^{131, 133} the cytotoxicity of the ELP-CLP vesicles was assessed with a murine embryonic fibroblast cell line (NIH-3T3). Cells were plated using standard cell culture procedures and incubated with preformed vesicles for 24 hours, after which they were subjected to live and dead stains (calcein AM and propidium iodide, respectively) and assessed for viability via fluorescence microscopy. Results for untreated cell controls are shown in Figure 6a and 6b and results for cells treated with 1000 µg/mL vesicles are shown in Figure 6c and 6d, respectively. Similar experiments were also performed for cells cultured with vesicles at lower concentrations (Figure S8). The phase-contrast images presented in Figure 6a and 6c and the fluorescence images in Figure 6b and 6d illustrate apparent healthy morphologies as well as high cell viability (lack of red fluorescence) for both the treated and untreated cells. The distinct similarities between the untreated control and the vesicle-treated cells suggest that the vesicles are non-toxic to murine fibroblast cells after 24 hours of exposure at high concentrations.

The lack of cytotoxicity of these vesicles is in good agreement with previous literature reports of the interaction of fibroblasts with peptide based nanoparticle systems. Specifically, ELP based nanoparticles have been shown to induce proliferation in human dermal fibroblasts as well as possess general viability with macrophages^{139, 143} and chondrocytes.¹⁴⁴ Similarly, a recent report of metal coordinated dipeptide nanoparticles showed that after incubation of these particles with fibroblasts for a 48 hour period, cells showed good viability according to a MTT proliferation assay.¹⁴⁵ In contrast, nanoparticles assembled from non-biological materials such as inorganic silver and gold nanoparticles have been shown to possess substantial cytotoxicity, affecting cell morphology, membrane integrity, and proliferation in the case of silver,^{146, 147} and motility, substrate adhesion, and proliferation for both human¹⁴⁸ and murine¹⁴⁹ fibroblasts in the case of gold.

3.3.3 Chondrocyte viability and proliferation—Considering the critical role of chondrocytes in producing and maintaining the collagen and proteoglycans matrix in the cartilage, the compatibility of the vesicles with ATDC5 chondrogenic cell line was investigated. ATDC5 cells are widely utilized as an *in vitro* model to study the factors that influence cell behaviors during chondrogenesis.¹⁵⁰ To determine the effect of ELP-CLP vesicle concentration on ATDC5 cells, changes in cell viability and proliferation were quantified via live/dead assay and cell growth, respectively, over time. ELP-CLP vesicles did not induce ATDC5 cell death at either 24 or 72 hours for any of the ELP-CLP concentrations tested (Figure S9), as evidenced by the fact that the percentage of live cells at all ELP-CLP concentrations and timepoints were not significantly different from that of untreated cells (Figure 7a). Additionally, cell proliferation did not appear to be influenced by ELP-CLP presence (Figure 8), as cell number (Figure 7b) and degree of areal cell coverage Figure S10) increased similarly across all groups with time.

Although our study did not investigate the effect of ELP-CLP vesicles on primary chondrocytes or intact cartilage tissues, our findings regarding the cytocompatibility of ELP-CLP vesicles with regard to chondrocyte health are consistent with recent literature assessing the effect other peptide and polymeric nanoparticle formulations on chondrocytes/cartilage *in vitro* and *in vivo*. Examples include: chitosan-,^{151–153} poly(propylene)-,¹⁵⁴ PLGA-,^{155, 156} poly-beta amino esters (PBAEs)-,¹⁵⁷ PNIPAM-,¹⁵⁸ and polysebacic anhydride-based¹⁵⁹ nanoparticles as well as HA-coated nanoparticles,^{160, 161} which have been utilized to improve outcomes ranging from the enhancing the tissue engineering of cartilage to modifying osteoarthritic disease. Additionally, both ELPs and CLPs have been successfully utilized, separately, as scaffold/templates for articular cartilage regeneration,^{162–166} further confirming their biocompatibility.

Taken together, the cargo encapsulation, targeting, and retention findings, in combination with the *in vitro* results in macrophage, fibroblast, and chondrocyte cell lines, suggest that ELP-CLP vesicles may be useful as viable, biocompatible, non-cytotoxic delivery vehicles for targeting collagen within the articular cartilage of diarthroidial joints, and thus a promising platform for the treatment of a variety of diseases with high collagen remodeling.

4. Conclusions

In this work, we demonstrated the potential of utilizing ELP-CLP vesicles as thermoresponsive drug delivery vehicles for targeting collagen-containing matrices. Physical encapsulation of fluorescein as a model hydrophobic drug did not alter the ability of the ELP-CLP to form nanosized vesicles. A sustained release of a clinically relevant amount of cargo was achieved over periods of 3 and 6 weeks. More importantly, a thermoresponsive burst release was observed by dissociating the vesicles above the unfolding temperature of the CLP domain, indicating the potential of using hyperthermia treatment for triggering the release of encapsulated drug from appropriately engineered ELP-CLPs. The ability of the ELP-CLP vesicles to bind with high affinity to type II collagen film suggests their use in collagen-targeting applications. The cytocompatibility of the ELP-CLPs with both NIH-3T3 fibroblasts and ATDC5 chondrocytes, coupled with the lack of TNF- α production by RAW264.7 macrophages, suggests the minimal inflammatory activity of the vesicles. Taken together, the collagen-targeting ability from the CLP domain, thermoresponsiveness from the ELP domain, controlled long-term cargo release, cytocompatibility, and lack of inflammation all support that ELP-CLP vesicles could be promising candidates as drug carriers for the localized treatment of a wide range of diseases associated with high collagen remodeling activity, such as joint disorders. Further studies will systematically evaluate the effect of peptide sequence and length on thermoresponsive drug release, specifically with respect to controlling the temperature ranges for nanovesicle assembly and dissociation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Dynamic light scattering characterization of the vesicles. a) Size distributions of cargo-free ELP-CLP assemblies. d) Size distributions of fluorescein-loaded ELP-CLP assemblies. (Figure a is reproduced/adapted from reference 44. Copyright 2015 American Chemical Society.)

Figure 2.

Transmission electron microscopy images of the vesicles. a) Cargo-free vesicles from ELP-CLP conjugates at 37 °C. b) Cargo-free ELP-CLP conjugates at 80 °C. c) Fluorescein-loaded vesicles from ELP-CLP conjugates at 37 °C. d) Fluorescein-loaded ELP-CLP conjugates at 80 °C. Scale bars = 500 nm. (Figure a and b are reproduced/adapted from reference 44. Copyright 2015 American Chemical Society.)

Figure 3. Cumulative release of fluorescein from ELP-CLP vesicles at physiological temperature (37 °C) as a function of time. The amount of released fluorescein was calculated by measuring the fluorescence at 535 nm using a fluorescein calibration curve and normalized to the total amount of encapsulated fluorescein. At day 21, the sample was heated at 80 °C before the measurement. N = 3.

Figure 4. Cumulative release of fluorescently-labelled FELP-CLP vesicles from type II collagen films at Day 0, Day 5, Day 10 and Day 10 after heating. The amount of released vesicles was calculated by measuring the fluorescence at 535 nm. At day 10, the film was heated at 80 °C before measurement. All measurements were performed at room temperature. N = 4 for the preincubated sample; N = 3 for the unpreincubated control.

Figure 5.

Activation of murine-derived RAW264.7 macrophages treated with ELP-CLP vesicles. RAW264.7 macrophages were cultured on TCPS and incubated with medium only (control), 50 ng/mL LPS, or various concentrations of ELP-CLP vesicles for 8 hours prior to evaluation of TNF- α secretion by macrophages. N=3. *represents significance from control cultures and cultures treated with vesicles at any concentration ($p<0.0001$; as determined via ANOVA with Tukey HSD posthoc).

Figure 6.

Cell viability of NIH-3T3 fibroblastic cells after 24 hour incubation with serum. All images are with a 10× objective. a,b) phase image and live/dead assay stained fluorescence image of 0 µg/mL vesicle control; c,d) phase image and live/dead assay stained fluorescence image of 1000 µg/mL vesicles. Scale bars = 100 µm.

Figure 7.

Quantitative analysis of ATDC5 viability and proliferation following application of ELP-CLP vesicles *in vitro*. a) ATDC5 cell viability (% live cells) and b) cell number following application of 0, 50, 150, 500, and 1000 µg/mL of ELP-CLP vesicles. Cell viability and proliferation is unaltered by application of ELP-CLP vesicles at any concentration. Results are presented as mean $\pm$ SD (N = 3-5/timepoint/concentration). *represents significance between any of the four groups ($p < 0.01$; as determined via ANOVA with Tukey HSD posthoc) and no significance within each group.

Figure 8.

ADTC5 cell proliferation in the presence of ELP-CLP vesicles *in vitro*. Cells were imaged via phase contrast microscopy at 24 hours and 72 hours post-ELP-CLP vesicles application (0 and 1000 $\mu\text{g/mL}$ shown at top and bottom panel, respectively). Application of ELP-CLP vesicles does not appear to cause ATDC5 death or changes in cell proliferation. Scale bar = 200 μm and applies to all images.

Scheme 1.

Chemical conjugation of ELP and CLP using CuAAC “click” reaction. (Reproduced/adapted from reference 44. Copyright 2015 American Chemical Society.)