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Use of methionine alkylation to prepare cationic and zwitterionic block copolypeptide vesicles

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Abstract

We have developed a facile method for preparation of ionic copolypeptide vesicles that requires no protecting groups or expensive components. We prepared amphiphilic copolypeptides containing segments of water soluble methionine sulfonium residues that were derived from a fully hydrophobic precursor diblock copolypeptide, poly(L-methionine)₆₅-*block*-poly(L-leucine_{0.5}-*stat*-L-phenylalanine_{0.5})₂₀, M₆₅(L_{0.5}/F_{0.5})₂₀, by its direct alkylation in water via reaction with simple alkylating reagents. Methylation of M₆₅(L_{0.5}/F_{0.5})₂₀ gave the cationic methyl-methionine sulfonium derivative, M^M₆₅(L_{0.5}/F_{0.5})₂₀, and carboxymethylation gave the zwitterionic carboxymethyl-methionine sulfonium derivative, M^C₆₅(L_{0.5}/F_{0.5})₂₀. Assembly of M^M₆₅(L_{0.5}/F_{0.5})₂₀ or M^C₆₅(L_{0.5}/F_{0.5})₂₀ in water gave rise to vesicles with average diameters of a few microns that could

then be extruded to nanoscale diameters. While the cationic M^M based vesicles were found to be cytotoxic, the zwitterionic M^C based vesicles were found to possess minimal cytotoxicity.

1. Introduction

Polymeric nanocarriers show great promise for delivery of therapeutics and encapsulation of cargos. For successful translation to applications, it is critical that these carriers incorporate many levels of functionality, such as cellular uptake or triggered disruption, which often requires use of complex chemistries and designs.^[1] Synthetic carriers prepared solely from natural components that are resorbable and biocompatible are desirable, yet preparation of such materials can be challenging.^[2] Here, we developed a method to prepare nanoscale vesicles composed of block copolypeptides using simple and economical chemistry based on the efficient, chemoselective, and broad scope alkylation of methionine residues.^[3] This chemistry allows nanocarriers to be prepared from inexpensive natural amino acids, and also provides a means for introduction of a wide range of useful chemical functionalities. This ability to simultaneously create amphiphilic block copolypeptides and add functionality to the resulting materials in a single, efficient step provides a useful method to create well-defined assemblies with multiple combinations of properties in a straightforward manner.

Block copolypeptide vesicles are promising nanocarriers possessing attractive features of biodegradability, tunable size and stability, and ability to incorporate the functionality of proteins.^[2,4] Our lab and others have previously reported vesicles composed of a variety of amphiphilic block copolypeptides possessing a variety of properties.^[5] However, many of these materials required sophisticated syntheses, including multistep monomer synthesis, use of protecting groups, or expensive components.^[5] Our lab has recently been interested in streamlining the preparation of functional block copolypeptides by developing scalable synthetic

methods for preparation of these materials.^[3,6] We recently reported the preparation of enzyme responsive copolyptide vesicles incorporating non-toxic, water soluble methionine sulfoxide, M^O, segments where enzymatic reduction of M^O residues caused changes in chain conformations and solubility that resulted in vesicle rupture and release of encapsulated cargos.^[7] These materials utilized cell compatible and degradable components and were found to be excellent substrates for ubiquitous intracellular reductase enzymes, providing a potential means for intracellular cargo release. Most importantly, these materials were prepared in a simple and efficient process that required use of no protecting groups and utilized inexpensive amino acid building blocks, which was achieved by taking advantage of the unique chemistry of methionine, M, residues.

Here, we sought to further explore the utility of M chemistry for synthesis of functional copolyptide materials through the chemical alkylation of M residues. Our lab recently reported the use of M alkylation as a facile means to reversibly or irreversibly introduce useful functionality and chemically reactive groups onto polypeptides.^[3,8] This work originated from pioneering studies of M alkylation in proteins, which were mainly focused on use of non-functional alkylating reagents to probe inhibition of enzyme active sites.^[9-11] We have found that M residues can undergo chemoselective, broad scope, highly efficient alkylation reactions in homo- and copolypeptides yielding stable sulfonium derivatives. These “M click” functionalizations are compatible with deprotection of other functional groups, use an inexpensive, natural amino acid, and allow the introduction of a diverse range of functional and reactive groups onto polypeptides.^[3,8] Here, we have demonstrated the utility of M alkylation chemistry for the facile preparation of model amphiphilic block copolypeptides that can form vesicle assemblies in water (Scheme 1).

Scheme 1 Schematic showing structure, chemical functionalization, and proposed self-assembly of $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ ($R = CH_3$) and $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ ($R = CH_2C(O)O^-$) block copolypeptides into vesicles.

2. Experimental

Materials and general procedures. Anhydrous tetrahydrofuran (THF), hexane and diethyl ether were prepared by passage through alumina columns, and oxygen was removed by purging with nitrogen prior to use. 1H NMR spectra were recorded on a Bruker AVANCE 400 MHz spectrometer. All Fourier Transform Infrared (FTIR) samples were prepared as thin films on NaCl plates with spectra recorded on a Perkin Elmer RX1 FTIR spectrometer, and are reported in terms of frequency of absorption (cm^{-1}). Tandem gel permeation chromatography/light scattering (GPC/LS) was performed on a SSI Accuflow Series III liquid chromatograph pump equipped with a Wyatt DAWN EOS light scattering (LS) and Optilab rEX refractive index (RI)

detectors. Separations were achieved using 10^5 , 10^4 , and 10^3\AA Phenomenex Phenogel $5\ \mu\text{m}$ columns using 0.10 M LiBr in DMF as the eluent at $60\ ^\circ\text{C}$. All GPC/LS samples were prepared at concentrations of 5 mg/mL. Millipore ($18\ \text{M}\Omega$) water was obtained from a Millipore Milli-Q Biocel A10 purification unit.

Synthesis. All α -amino acid-N-carboxyanhydride (NCA) monomers were synthesized using previously described protocols. L-Phenylalanine and L-leucine NCAs were synthesized by phosgenation and purified by recrystallization.^[12,13] L-Methionine NCA was prepared by phosgenation and purified by anhydrous column chromatography.^[6] α -Methoxy- ω -isocyanatoethyl-poly(ethylene glycol)₄₅ (mPEG₄₅-NCO) was prepared by reacting α -methoxy- ω -aminoethyl-poly(ethylene glycol)₄₅ (mPEG₄₅-NH₂, $M_n = 2000\ \text{g mol}^{-1}$, Nanocs) with phosgene in THF for 16 h.^[6] All block copolypeptides were synthesized using $(\text{PMe}_3)_4\text{Co}$ initiator,^[14] and the synthesis of $M_{65}(\text{L}_{0.5}/\text{F}_{0.5})_{20}$ has been previously described.^[7]

Synthesis of poly(L-methionine)₆₅-block-poly(L-leucine_{0.5}-stat-L-phenylalanine_{0.5})₂₀, $M_{65}(\text{L}_{0.5}/\text{F}_{0.5})_{20}$. Met NCA (80 mg, 0.4 mmol) was dissolved in THF (1.6 mL) and placed in a 20 mL scintillation vial containing a stir bar. To the vial, $(\text{PMe}_3)_4\text{Co}$ initiator solution (280 μL of a 20 mg/mL solution in THF) was added via syringe. The vial was sealed and allowed to stir in the glove box for 1 h. An aliquot (20 μL) was removed and analyzed by FTIR to confirm that all the NCA was consumed. In the glove box, α -methoxy- ω -isocyanatoethyl-poly(ethylene glycol)₄₅ (mPEG₄₅-NCO) (20 mg) was dissolved in THF (1 mL) in a 20 mL scintillation vial. An aliquot (240 μL) of the polymerization solution containing active chain ends was removed and added to the solution of mPEG₄₅-NCO. This PEG end-capping reaction was sealed and allowed to stir for 24 h. L-Leucine-N-carboxyanhydride (Leu NCA) (10.5 mg, 0.07 mmol) and L-phenylalanine-N-carboxyanhydride (Phe NCA) (12.8 mg, 0.07 mmol) were dissolved separately in THF (210 μL

and 260 μL , respectively), combined and added to the remaining polymerization solution via syringe. The vial was sealed and allowed to stir in the glove box for 1 h to give the diblock $\text{M}_{65}(\text{L}_{0.5}/\text{F}_{0.5})_{20}$. FTIR was used to confirm complete consumption of NCAs. Outside the glove box, the block copolyptide was isolated by evaporation of all volatiles and washing the residue in 10 mM HCl (3 times) to remove all cobalt ions. The white precipitate was washed with two portions of DI water and then lyophilized to yield $\text{M}_{65}(\text{L}_{0.5}/\text{F}_{0.5})_{20}$ as a fluffy white solid (93 % yield).

Outside of the glove box, the PEG end-capped sample ($\text{M}_x\text{-PEG}_{45}$) was treated with HCl (2 equiv per $(\text{PMe}_3)_4\text{Co}$, 6 M in H_2O). After 10 min stirring, $\text{M}_x\text{-PEG}_{45}$ was collected by precipitation into acidic water (pH 3, HCl, $>10\times$ the reaction volume), followed by centrifugation. The solid was washed with water (3 times) to remove all unconjugated mPEG₄₅-NCO. $\text{M}_x\text{-PEG}_{45}$ was placed under high vacuum to remove residual H_2O before NMR analysis. Since it has been shown that end-capping is quantitative for $(\text{PMe}_3)_4\text{Co}$ initiated NCA polymerizations when excess isocyanate is used,^[15] integrations of methionine resonances versus the polyethylene glycol resonance at δ 3.64 could be used to obtain the M segment length.^[6] The average composition of the copolymer was then determined by ^1H NMR integrations of methionine resonances and comparison to leucine and phenylalanine resonances. The actual diblock copolyptide composition was found to be $\text{M}_{66}(\text{L}_{0.5}/\text{F}_{0.5})_{22}$, and is denoted as $\text{M}_{65}(\text{L}_{0.5}/\text{F}_{0.5})_{20}$.

Preparation of poly(S-methyl-L-methionine sulfonium chloride)₆₅-block-(L-leucine_{0.5}-stat-L-phenylalanine_{0.5})₂₀, $\text{M}_{65}^{\text{M}}(\text{L}_{0.5}/\text{F}_{0.5})_{20}$. A 20 mL scintillation vial was charged with $\text{M}_{65}(\text{L}_{0.5}/\text{F}_{0.5})_{20}$ (10 mg), H_2O (500 μL) and a stir bar. Iodomethane (8 μL , 2 eq. per M residue) was added to the copolyptide suspension via syringe. The reaction was sealed, covered in foil and stirred for 48

hours at room temperature. The resulting mixture was transferred to a 2000 MWCO dialysis bag and dialyzed against Millipore water containing sodium bisulfite for 24 hours with 3 dialyzate changes. To exchange all counterions to chloride, the copolymer was dialyzed against NaCl (200 mM in Millipore water) for 24 hours with 3 dialyzate changes, and then dialyzed against Millipore water with 3 dialyzate changes. The copolypeptide suspension was lyophilized to yield the product as a white solid. The yield was 80 % (some sample was lost by leakage from dialysis bag) of 100 % functionalized copolymer.

Preparation of poly(S-carboxymethyl-L-methionine sulfonium)₆₅-block-(L-leucine_{0.5}-stat-L-phenylalanine_{0.5})₂₀, M^C₆₅(L_{0.5}/F_{0.5})₂₀. A 20 mL scintillation vial was charged with M₆₅(L_{0.5}/F_{0.5})₂₀ (10 mg), H₂O (500 μL) and a stir bar. Iodoacetic acid (43 mg, 4 eq. per Met residue) was dissolved in H₂O (860 μL, 50 mg/mL) and added to the copolypeptide suspension via syringe. The reaction was sealed, covered in foil and stirred for 48 hours at room temperature. The solution was transferred to a 2000 MWCO dialysis bag and dialyzed against Millipore water containing sodium bisulfite for 24 hours with 3 dialyzate changes. The copolymer was dialyzed against NaCl (200 mM in Millipore water) for 24 hours with 3 dialyzate changes, and then dialyzed against Millipore water with 3 dialyzate changes. The copolypeptide suspension was lyophilized to yield the product as a white solid. The yield was 80 % (some sample was lost by leakage from dialysis bag) of 100 % functionalized copolymer.

Preparation of M^M₆₅(L_{0.5}/F_{0.5})₂₀ and M^C₆₅(L_{0.5}/F_{0.5})₂₀ vesicle assemblies. Solid copolypeptide powder was dispersed in THF to give a 4 % (w/v) suspension, which was then placed in a bath sonicator for 30 minutes until the copolypeptide was evenly dispersed. An equal volume of Millipore water was added to the suspension, which was then placed in a bath sonicator for 30 minutes. Four equivalent aliquots of THF were then added in succession to the suspension, with

vortexing in between each addition, to give a final sample concentration of 1 % (w/v) and a 3:1 ratio of THF to water. The resulting suspension was then placed in a dialysis bag (MWCO = 2000 Da) and dialyzed against Millipore water under sterile conditions for 24 hours. The dialyzate was changed every hour for the first 4 hours.

Fluorescent probe conjugation to $M_{65}(L_{0.5}/F_{0.5})_{20}$. 5-(Iodoacetamido)fluorescein was conjugated to thioether groups of methionine side chains via an alkylation reaction.^[7] $M_{65}(L_{0.5}/F_{0.5})_{20}$ (10 mg) was dissolved in DMF (1 mL) in a 20 mL scintillation vial. 5-(iodoacetamido)fluorescein was dissolved in DMF (10 mg/mL) and added to the 1 % (w/v) copolypeptide suspension at a 5:1 molar ratio of fluorescent probe to copolypeptide chain (i.e. 5 probes per every 65 M residues). The alkylation was allowed to proceed for 16 h in the dark, followed by evaporation of the DMF under vacuum. After fluorescein modification, the remaining methionine residues were then alkylated to methionine sulfoniums as described above.

Dye Encapsulation in Polypeptide Vesicles. The diblock copolypeptide $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ was dispersed in THF to give a 4 % (w/v) suspension, which was then placed in a bath sonicator for 30 minutes until the copolypeptide was evenly dispersed. An equal volume of Millipore water containing Texas Red labeled dextran (Molecular Probes, MW = 3000 Da, 0.25 mg/mL) was added to the suspension to give a final sample concentration of 2 % (w/v), and the mixture was then placed in a bath sonicator for 30 minutes. Four equivalent aliquots of THF were then added in succession to the suspension, with vortexing in between each addition, to give a final sample concentration of 1 % (w/v) and a 3:1 ratio of THF to water. The suspension was then placed in a dialysis bag (MWCO = 2000 Da) and dialyzed against Millipore water under sterile conditions for 24 hours in the dark, with the dialyzate changed every hour for the first 4 hours. The resulting suspension was then transferred to a new dialysis bag (MWCO = 8000 Da), and was dialyzed for

24 hours to remove all dextran that was not encapsulated by the vesicles. The dialyzate was changed every hour for the first 4 hours.

Differential interference contrast microscopy (DIC). Suspensions of copolypeptide vesicles (0.5 % (w/v)) were visualized on glass slides with a spacer between the slide and the coverslip (either double-sided tape or a Secure Seal Imaging Spacer, *Grace Bio-labs*) allowing the self-assembled structures to be minimally disturbed during focusing. The samples were imaged using a Zeiss Axiovert 200 DIC/Fluorescence Inverted Optical Microscope.

Extrusion of copolypeptide vesicles. Aqueous vesicle suspensions composed of $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ or $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ were diluted to 0.2 % (w/v) and extruded using an Avanti Mini-Extruder. Extrusions were performed using different pore size Whatman Nucleopore Track-Etched polycarbonate (PC) membranes, following a protocol of serial extrusion through decreasing filter pore sizes: 3 times through a 1.0 μm filter, 3 times through a 0.4 μm filter, 3 times through a 0.2 μm filter, and 3 times through a 0.1 μm filter. The PC membranes and filter supports were soaked in Millipore water for 10 minutes prior to extrusion.

Dynamic light scattering (DLS) analysis of extruded vesicles. Extruded aqueous copolypeptide vesicle suspensions at 0.2 % (w/v) were placed in disposable cuvettes and analyzed using a Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc, Westborough, MA). A total scattering intensity of approximately 1×10^5 cps was targeted.

Zeta potential analysis of copolypeptide vesicles. A 0.5 % (w/v) aqueous suspension of either extruded $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ or $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ vesicles was diluted to 0.2 % (w/v) with Millipore water containing NaCl to give a final concentration of 10 mM salt. The pH was then adjusted using NaOH or HCl to give suspensions ranging from pH 3 to pH 9. The suspensions were then

placed in disposable capillary cells (Malvern Instruments Inc, Westborough, MA). Zeta potentials were measured with the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc, Westborough, MA).

Laser scanning confocal microscopy (LSCM) of fluorescently labeled vesicles. LSCM images of copolypeptide vesicles were taken on a Leica Inverted TCS-SP1 MP-Inverted Confocal and Multiphoton Microscope equipped with an argon laser (476 and 488 nm blue lines), a diode (DPSS) laser (561 nm yellow-green line), and a helium-neon laser (633 nm far red line). Suspensions of the fluorescently labeled copolypeptide vesicles (0.5 % (w/v)) were visualized on glass slides with a spacer between the slide and the cover slip (Secure Seal Imaging Spacer, *Grace Bio-labs*) allowing the self-assembled structures to be minimally disturbed during focusing. Imaging of an xy plane with an optical z-slice (700 nm) showed that the assemblies were water filled, unilamellar vesicles.

Transmission electron microscopy (TEM) of extruded vesicles. Extruded aqueous suspensions of copolypeptide vesicles were diluted to 0.1 % (w/v) with Millipore water. Samples (4 μ L) were placed on 300 mesh Formvar/carbon coated copper grids (Ted Pella) and allowed to remain on the grid for 60 seconds. Filter paper was used to remove residual fluid. One drop of 2 % (w/v) uranyl acetate (negative stain) was then placed on each grid for 90 seconds, and subsequently removed by washing with drops of Millipore water and removing the excess liquid with filter paper. Sample containing grids were allowed to dry in air before imaging with a JEM 1200-EX (JEOL) transmission electron microscope at 80 kV.

Measurement of copolypeptide vesicle suspension concentrations. The Bradford assay was performed to quantify the final concentrations of copolypeptide vesicle suspensions after

extrusion. The assay was performed according to the manufacture supplied instructions, using known concentration samples as standards.

Cell culture. The HeLa cell line was grown in Minimal Essential Medium supplemented with 26.2 mM sodium bicarbonate, 1 mM sodium pyruvate, 10% FBS, and 1% penicillin/streptomycin, at a pH of 7.4. The cell line was maintained in a 37 °C humidified atmosphere with 5% CO₂ and handled with standard sterile tissue culture protocols.

Measurement of vesicle cytotoxicity using the MTS cell proliferation assay. The MTS cell proliferation assay (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay) was performed to assess cell viability after exposure to copolypeptide vesicles. The cytotoxicity studies were performed using HeLa cells seeded on 96-well plates with triplicates of each condition. After cells were incubated with vesicle samples for 5 h at 37 °C, the medium was aspirated and fresh medium containing 20% MTS reagent was added to the cells. The cells were incubated again at 37 °C for 1 h, and then their absorbance at 490 nm and 700 nm were measured using an Infinite F200 plate reader (Tecan Systems Incorporated, San Jose, CA). The relative survival of cells compared to control cells (*i.e.*, cells incubated in growth medium without vesicles) was calculated by determining the ratio of the (A₄₉₀ – A₇₀₀) values.

3. Results and Discussion

Katchalski was the first to alkylate poly(L-methionine), preparing both methyl and carboxymethyl sulfonium derivatives from the corresponding alkyl bromides and poly(L-methionine) in neat formic acid. As opposed to α -helical, hydrophobic poly(L-methionine), these polysulfoniums were found to be stable, water soluble, and were studied for their conformational and polyelectrolyte behavior.^[16] Despite these promising early results, M alkylation has not been previously used with block copolypeptides. Here, we sought to use this

versatile chemistry as a means to prepare ionic amphiphilic block copolypeptides in a simple, economical process, and to demonstrate its utility as a potential means to prepare functional polypeptide based assemblies.

While other synthetic peptide based assemblies require that biofunctional groups (e.g. peptides, functional side chains) either be protected during synthesis or conjugated to a material post-polymerization,^[5] alkylated M containing amphiphilic copolypeptides were readily prepared by polymerization of inexpensive, natural, hydrophobic amino acid building blocks. Initially, a fully hydrophobic precursor diblock copolypeptide, poly(L-methionine)₆₅-b-poly(L-leucine_{0.5}-stat-L-phenylalanine_{0.5})₂₀, M₆₅(L_{0.5}/F_{0.5})₂₀, was synthesized via cobalt catalyzed living polymerization of the corresponding *N*-carboxyanhydride monomers (Scheme 1, see SI, Table S1).^[7] The segment lengths were based on those previously found to promote vesicle formation using other amino acids, i.e. an approximately 3:1 ratio of hydrophilic to hydrophobic segment lengths,^[5,12,13] and phenylalanine was incorporated within the rod-like, permanently hydrophobic leucine segment to enhance membrane flexibility.^[17] Subsequent alkylation of this hydrophobic precursor in water, using either iodomethane or iodoacetic acid as model alkylating agents, directly gave the desired amphiphilic sulfonium derivatives, M^M₆₅(L_{0.5}/F_{0.5})₂₀ or M^C₆₅(L_{0.5}/F_{0.5})₂₀, in high yield and purity, respectively (Scheme 1, see SI). In addition, due to its use in animal feeds, more methionine is produced chemically than any other amino acid,^[18] which lowers its cost and makes this route a very economical process for synthesis of ionic amphiphilic copolypeptides.

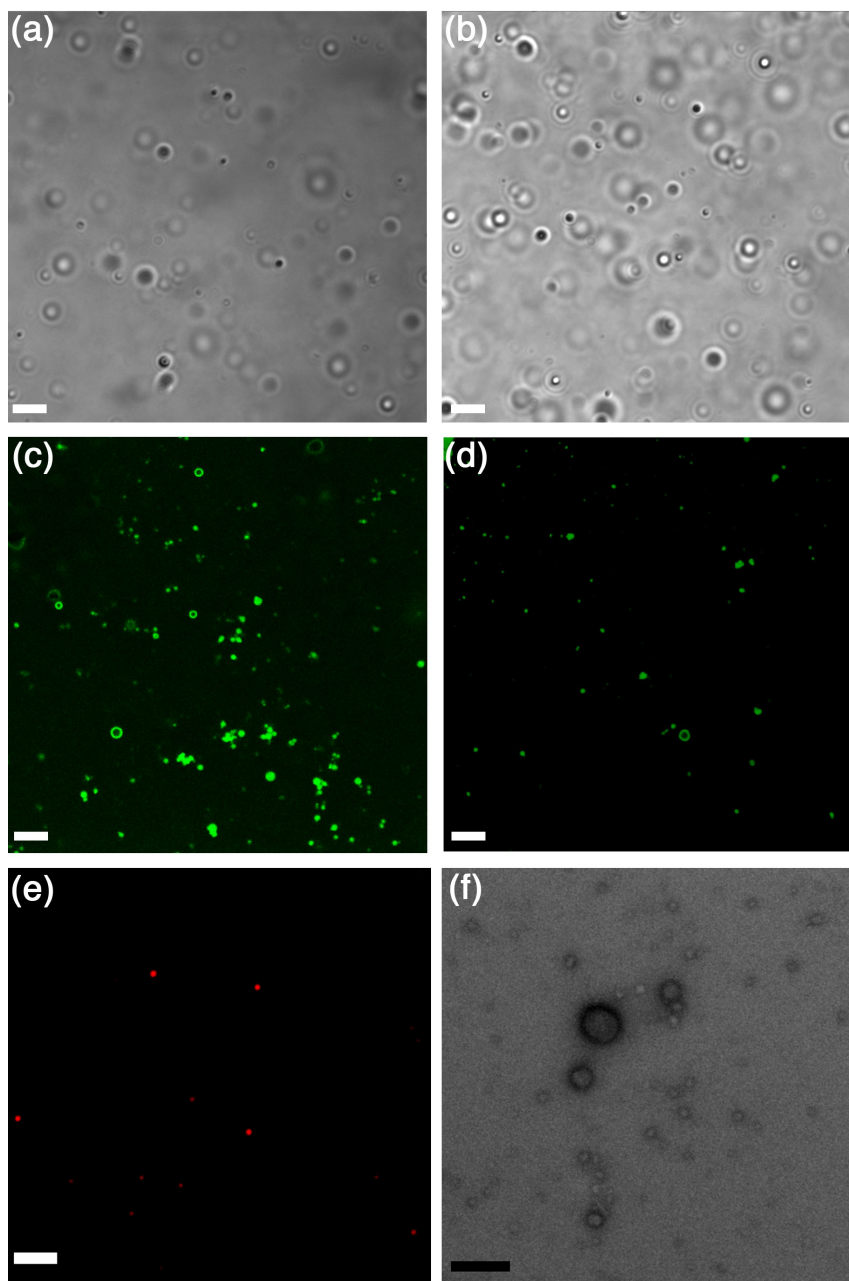


Figure 1 Images of methionine sulfonium containing copolyptide vesicles. (a) DIC image of a 1 % (w/v) $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ aqueous vesicle suspension. (b) DIC image of a 1 % (w/v) $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ aqueous vesicle suspension. (c) LSCM image of a fluorescein labeled 1 % (w/v) $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ aqueous vesicle suspension. (d) LSCM image of a fluorescein labeled 1 % (w/v) $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ aqueous vesicle suspension. (e) LSCM image of a 1 % (w/v) $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ aqueous vesicle suspension encapsulating Texas Red dextran. (f) TEM image of negatively

stained 0.1 % (w/v) $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ aqueous 0.1 μm extruded vesicle suspension. Scale bars: a-e = 5 μm ; f = 400 nm.

Assembly of either $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ or $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ in water using mixed solvent annealing followed by dialysis gave polydisperse, unilamellar vesicles with average diameters of a few microns,^[5,12] as determined by optical microscopy and laser scanning confocal microscopy (LSCM) (Figure 1a-d). These structures are consistent with results obtained for many other block copolypeptide amphiphiles of similar block composition,^[5,12] validating the chosen sequence design. Similar to previous work,^[5,12] these new vesicles were found to be capable of encapsulating polar cargos, such as Texas Red labeled dextran ($M_n = 3000$ Da) (Figure 1e). For applications where nanoscale diameters are required, the vesicles can also be reduced in size by extrusion through polycarbonate filters to give nanoscale vesicles with average diameters of *ca.* 100 to 150 nm as determined by TEM, and dynamic light scattering (DLS) (Figure 1f, see SI, Table S2). Overall, these alkylated M segments were found to serve as excellent hydrophilic domains for preparation of stable, ionic copolypeptide vesicles of controllable size.

Both $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ and $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ vesicles formed stable suspensions that did not aggregate or precipitate over time in aqueous media. The cationic nature of the M^M segments, and the zwitterionic nature of the M^C segments were confirmed by zeta potential measurements on vesicle suspensions. The $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ vesicles possessed highly positive zeta potentials across a broad pH range, while the $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ vesicles possessed slightly negative zeta potentials above pH 3, with a shift toward positive zeta potential as pH was decreased to 3 (Figure 2). These results are consistent with the molecular structures, where the methyl-methionine sulfonium of M^M is a cationic species that has no active protons within the pH range studied. The M^C vesicles also contain pH invariant cationic sulfonium groups, but also contain

anionic carboxylate groups that can be protonated at low pH, making this a zwitterionic segment above pH 3, that can transition to a cationic segment at lower pH. At neutral pH, these model vesicles, $M^M_{65}(L_{0.5}/F_{0.5})_{20}$ and $M^C_{65}(L_{0.5}/F_{0.5})_{20}$, can be considered to be cationic and zwitterionic, respectively.

Figure 2 Plot of zeta potential vs. pH for 0.2 % (w/v) $M^M_{65}(L_{0.5}/F_{0.5})_{20}$ (triangles) and $M^C_{65}(L_{0.5}/F_{0.5})_{20}$ (squares) suspensions containing 10 mM NaCl. Error bars represent the standard deviation from an average of three measurements.

Since the overall charge of copolypeptide vesicles has been shown to strongly influence their interactions with cells, we performed viability measurements on HeLa cells exposed to increasing concentrations of $M^M_{65}(L_{0.5}/F_{0.5})_{20}$ or $M^C_{65}(L_{0.5}/F_{0.5})_{20}$. Extruded cationic $M^M_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles were found to be toxic to HeLa cells in a concentration dependent manner (Figure 3), similar to results obtained with other cationic copolypeptide vesicles.^[12,13] These results show that the cationic sulfonium groups introduced via M alkylation can lead to cytotoxicity in resulting materials. However, extruded zwitterionic $M^C_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles were found to be non-toxic to HeLa cells at concentrations up to 100 $\mu\text{g}/\text{mL}$ (Figure 3), similar to properties observed with

other zwitterionic polymer systems.^[19] These results show that although potentially toxic cationic sulfonium groups are introduced via M alkylation, these effects can be eliminated by simultaneous introduction of charge neutralizing functionality, as in the case of carboxymethylation.

Figure 3 Viability of HeLa cells after incubation with extruded $M_{65}^M(L_{0.5}/F_{0.5})_{20}$ (triangles) or $M_{65}^C(L_{0.5}/F_{0.5})_{20}$ (squares) copolypeptide vesicles as functions of copolypeptide concentration relative to HeLa cells without polypeptide. Cells were incubated with vesicles for 5 h and cell viability was determined using the MTS assay relative to a cell only control. Relative survival = the ratio of sample survival to control survival. Error bars represent the standard deviation from an average of three measurements.

4. Conclusions

Here we have shown that use of M segments in block copolypeptides imparts these materials with the ability to be readily converted via alkylation into amphiphilic copolymers capable of self-assembly into ordered structures. The copolypeptides can also be functionalized by addition

of a variety of different groups during the alkylation reactions, as has been previously described.
[3] In particular, we have shown in proof of concept studies that either cationic or zwitterionic copolyptide vesicles can be prepared from a common precursor, and the resulting materials possess substantially different *in vitro* cytotoxicity toward HeLa cells. The economical synthesis and tunable properties of alkylated M containing block copolyptides make these materials attractive for many synthetic material challenges.

Supporting Information Polymer characterization, DLS data, and spectral data. Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/ijch.2010xxxxx>.

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