Triggered inversion of dual responsive diblock copolypeptide vesicles

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Abstract

We report the synthesis of amphiphilic poly(L-methionine sulfoxide)$_x$-*b*-poly(dehydroalanine)$_y$, diblock copolypeptides, $\mathbf{M^O_xA^{DH}_y}$, and their self-assembly into sub-micron diameter, unilamellar vesicles in aqueous media. Formation of vesicles was observed over an unprecedented range of copolypeptide compositions due to the unique properties and chain conformations of $\mathbf{A^{DH}}$ hydrophobic segments. These copolypeptides incorporate two distinct thiol reactive components, where each segment can respond differently to a single thiol stimulus. Incubation of $\mathbf{M^O_{35}A^{DH}_{30}}$ vesicles with glutathione under intracellular mimetic conditions resulted in vesicle disruption and release of cargo. Further, incubation of $\mathbf{M^O_{35}A^{DH}_{30}}$ vesicles with thiolglycolic acid resulted in reversal of amphipilicity and successful *in situ* inversion of the vesicle assemblies. This conversion of biomimetic polymer vesicles into stable inverted vesicles using a biologically relevant stimulus at physiological pH and temperature is unprecedented. These results provide insights toward development of advanced functional synthetic assemblies with potential uses in biology and medicine.

Introduction

There is considerable interest in polymer assemblies that can undergo changes in structure and properties in response to biologically relevant stimuli.[1-3] Appropriate stimuli can include redox reagents, enzymes, sugars, as well as molecules such as NO, CO_2 and H_2S.[1-3] Such responsive assemblies have primarily been developed for use as therapeutic delivery vehicles that can release their cargo in the presence of an appropriate stimulus.[4-6] Consequently, many of these assemblies have been designed to simply degrade or disassemble upon reaction with the stimulus. However, block copolymer assemblies have also been developed that are able to undergo changes in shape and morphology upon exposure to stimuli.[7-9] In addition, 'invertible' assemblies have been developed where both block copolymer segments respond to stimuli resulting in reversal of amphiphilicity and consequent inversion of micellar or vesicular structures.[10-17]

Invertible assemblies are intriguing since they can release cargos, but can also allow for encapsulation of surrounding media during inversion. While a variety of shape changing transformations have been reported for assemblies under conditions that are not compatible with living systems, there are noteworthy exceptions. Vesicle-micelle transformations have been achieved using redox and hydrolysis reactions as well as enzymes,[1,2,13] and micelle-micelle inversions have been accomplished using light and/or heat.[11,15] However, to our knowledge, block copolymer vesicle to vesicle inversion has not been achieved under biologically relevant conditions, which would be valuable for the development of advanced functional synthetic assemblies as well as for potential uses in biology and medicine. Addressing this need, we describe the development of amphiphilic diblock copolypeptide vesicles where each polypeptide segment responds differently to a single biomimetic stimulus, resulting in vesicle inversion under biologically relevant conditions.

Pioneering examples of block copolymer vesicle inversion were reported by Eisenberg[12] and Lecommandoux.[14] Both approaches utilized a combination of acidic and basic segments in

block copolymers where at low pH acidic segments are neutral and basic segments are charged, and at high pH acidic segments are charged and basic segments are neutral. Reversal of amphiphilicity upon switching pH between 4 and 10 or 1 and 14 resulted in inversion of vesicle assemblies. At pH 7, both acidic and basic segments in these systems were charged and vesicles did not form. While these studies were the first to demonstrate inversion of polymeric vesicles, their potential applications were limited by the extremes in pH required for vesicle interconversion and stability. Consequently, we sought to develop self-assembled block copolymer vesicles that would be stable and capable of triggered inversion under biologically relevant conditions (e.g. pH 7.4, 150 mM NaCl, 37 ºC). These conditions preclude use of a large variation of temperature or solution pH to trigger inversion, but do allow use of reactive molecules that are found in biological systems.[1-3] We focused our vesicle design on block copolypeptides since both their chain conformations and side-chain functionality can be predictably manipulated.[18,19] If used for therapeutic applications, the peptide backbone also allows for enzymatic degradation *in vivo*.[20-21]

Ordered chain conformations in polypeptides can guide structure formation during assembly, a feature which cannot be readily replicated in most other synthetic polymers.[18,19] Many studies, by our lab and others, have led to a detailed understanding of how polypeptide chain conformations and block compositions influence structure formation and properties in aqueous based assemblies.[18,19] This work has resulted in robust designs for creation of block copolypeptide micelles,[22,23] vesicles,[24-26] and fibril based hydrogels[27-29] utilizing readily obtained α-helical, β-sheet, and disordered chain conformations. Block copolypeptide assemblies have also been developed that can respond to various biologically relevant stimuli. In particular, there has been considerable interest in redox responsive copolypeptide assemblies that can release cargo intracellularly by reduction of one segment using thiols or enzymes present within cell cytosol.[1-3,26] However, to obtain invertible assemblies, both segments in block copolypeptides need to be stimuli responsive to achieve switching of water solubility in both domains. To accomplish this,

we envisioned the incorporation of two distinct thiol reactive polypeptide segments in block copolymers, where each segment would respond differently to the single stimulus. Upon reaction with thiols, each segment would undergo a change in water solubility while simultaneously also undergoing a conformational change. These combined changes, in conjunction with optimization of copolymer compositions, was envisioned to yield invertible vesicles under biologically relevant conditions.

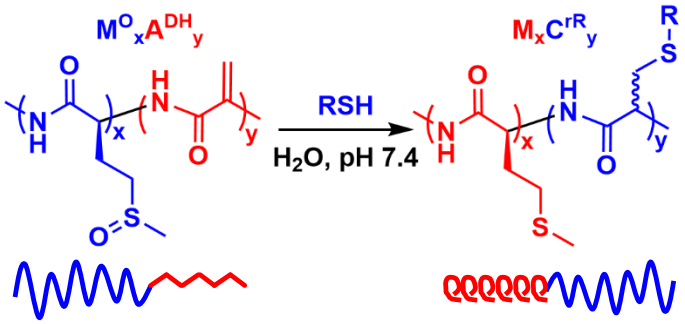

Figure 1. Proposed reversal of block copolypeptide amphiphilicity by reaction of $\mathbf{M^O_xA^{DH}_y}$ with thiols (endgroups not shown). Blue = hydrophilic; red = hydrophobic.

Our design of thiol reactive amphiphilic block copolypeptides incorporated water soluble, conformationally disordered poly(L-methionine sulfoxide), $\mathbf{M^O}$, segments connected to water insoluble, extended conformation poly(dehydroalanine), $\mathbf{A^{DH}}$, segments, $\mathbf{M^O_xA^{DH}_y}$ (Figure 1). Due to their amphiphilicity, $\mathbf{M^O_xA^{DH}_y}$ were expected to self-assemble in aqueous media. We have previously reported that $\mathbf{M^O}$ is highly biocompatible and can be used as a hydrophilic segment for preparation of vesicle and hydrogel block copolypeptide assemblies.[20,21] We have also shown that $\mathbf{M^O}$ segments can react with thiols or enzymes under conditions that mimic a cytosolic environment to form hydrophobic, α-helical poly(L-methionine), **M**, segments, and that this reaction can lead to vesicle disruption.[26] Additionally, our group recently reported the preparation of hydrophobic $\mathbf{A^{DH}}$, and showed that the dehydroalanine residues can react readily with thiols to give disordered poly(S-alkyl-*rac*-cysteine)s, $\mathbf{C^{rR}}$.[30] If a hydrophilic thiol is used, the resulting $\mathbf{C^{rR}}$ can be water soluble.[30] Individually, both of these polypeptide modifications are known to go to completion in aqueous media at pH 7.4.[26,30] Combination of $\mathbf{M^O}$ and $\mathbf{A^{DH}}$ segments in $\mathbf{M^O_xA^{DH}_y}$

block copolypeptides was expected to result in both segments being able to react with thiols under biologically relevant conditions. Further, complete reaction of $\mathbf{M^{O}_{x}A^{DH}_{y}}$ with a hydrophilic thiol should result in a reversal of amphiphilicity where hydrophilic $\mathbf{M^{O}}$ is converted to hydrophobic **M**, while hydrophobic $\mathbf{A^{DH}}$ is converted to hydrophilic $\mathbf{C^{rR}}$ (Figure 1).

While the design rules for aqueous self-assembly of amphiphilic block copolypeptides containing α-helical or β-sheet hydrophobic segments are well understood,[18,19] the self-assembly of $\mathbf{A^{DH}}$ containing block copolymers has not been previously studied. Here, an important goal was to determine how the unique chain conformation of $\mathbf{A^{DH}}$ influences structure formation in $\mathbf{M^{O}_{x}A^{DH}_{y}}$ assemblies.[30] We then planned to use this knowledge to design copolypeptide compositions able to form vesicles in water. An additional challenge of this project was the synthesis of $\mathbf{M^{O}_{x}A^{DH}_{y}}$, which requires the use of two different post-polymerization modification reactions that can interfere with each other. Here, we describe the successful preparation of $\mathbf{M^{O}_{x}A^{DH}_{y}}$ block copolypeptides, their self-assembly into vesicles in aqueous media, and how the vesicle assemblies respond to the addition of thiols under biologically relevant conditions. This work breaks new ground in the development of biologically relevant stimuli responsive copolymer assemblies by utilizing a combination of naturally occurring thiol-reactive amino-acid functionalities. The results also provide insights on understanding and controlling stimulus responsive switching of assemblies as mimics of protein assemblies.

Results and Discussion

Our initial strategy to prepare $\mathbf{M^{O}_{x}A^{DH}_{y}}$ copolypeptides relied on the stepwise addition of monomers L-methionine N-carboxyanhydride (Met NCA) and S-(*tert*-butylcarboxymethyl)-L-cysteine (*t*BuCM-Cys) NCA to $Co(PMe_3)_4$ initiator,[31] which gave poly(L-methionine)-*b*-poly(S-(*tert*-butylcarboxymethyl)-L-cysteine), $\mathbf{M_{x}C^{BCM}_{y}}$, diblock copolypeptide precursors of desired compositions and chain lengths. (see supporting information (SI) Figure S1). *tert*-Butyl protecting groups were subsequently removed from this precursor, and then all thioether groups were

oxidized to give samples of poly(L-methionine sulfoxide)-*b*-poly(S-(carboxymethyl)-L-cysteine sulfoxide), $\mathbf{M^{O}_{x}C^{CMO}_{y}}$. These copolymers were then subjected to a variety of conditions to convert S-(carboxymethyl)-L-cysteine sulfoxide residues to dehydroalanine residues via thermal or base catalyzed elimination in order to obtain $\mathbf{M^{O}_{x}A^{DH}_{y}}$ copolypeptides (see Figure S1). While there is precedent for conversion of isolated alkyl cysteine sulfoxide residues into dehydroalanine residues using sulfenic acid trapping reagents,[32] we were unable to obtain the desired $\mathbf{M^{O}_{x}A^{DH}_{y}}$ copolypeptides via this route.

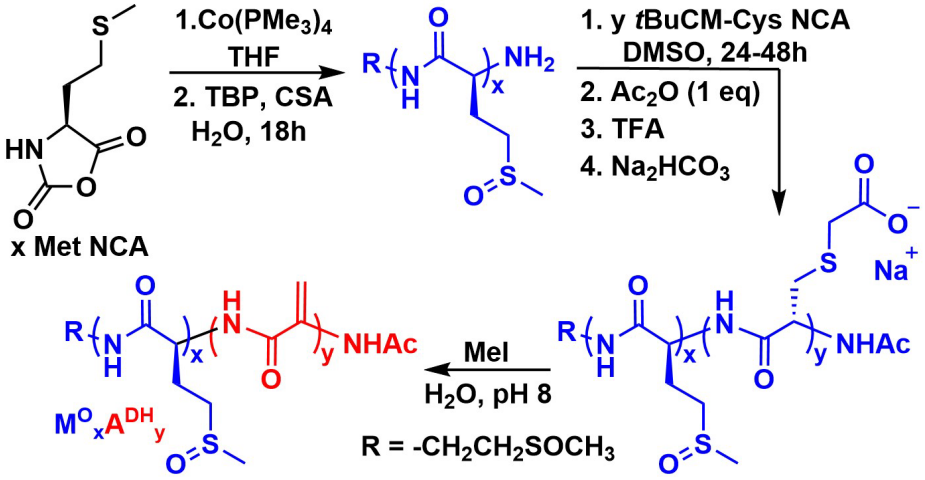

Figure 2. Synthesis of amphiphilic $\mathbf{M^{O}_{x}A^{DH}_{y}}$. Blue = hydrophilic; red = hydrophobic.

As an alternative, we have shown that alkylation of thioether groups in poly(S-(carboxymethyl)-L-cysteine), $\mathbf{C^{CM}}$, is highly efficient in promoting elimination and formation of $\mathbf{A^{DH}}$.[30] However, alkylation of a $\mathbf{M_{x}C^{CM}_{y}}$ precursor would also alkylate the **M** segment, and consequently prohibit its subsequent oxidation to $\mathbf{M^{O}}$.[33] While nucleophilic dealkylation of methionine sulfonium salts is possible,[34] the reagents, usually thiols, would also react with $\mathbf{A^{DH}}$ residues.[30] To circumvent these interfering reactions, we developed a new synthetic route to $\mathbf{M^{O}_{x}A^{DH}_{y}}$ copolypeptides that separates the oxidation and alkylation reactions so that they only occur within the desired segments (Figure 2). First, Met NCA was added to $Co(PMe_3)_4$ initiator to give **M** chains of desired length, which were then converted to $\mathbf{M^{O}}$ via oxidation. The $\mathbf{M^{O}}$ chains, each containing a primary amine group on the N-terminus, were then used as macroinitiators for

polymerization of *t*BuCM-Cys NCA in DMSO. The amino termini of the resulting block copolymers were end-capped using acetic anhydride,[30] followed by removal of *tert*-butyl protecting groups and then neutralization to give $\mathbf{M^{O}_{x}C^{CM}_{y}}$ copolypeptides. The thioether groups in $\mathbf{M^{O}_{x}C^{CM}_{y}}$ were then alkylated using methyl iodide, which resulted in facile elimination of the thioether byproduct to give $\mathbf{M^{O}_{x}A^{DH}_{y}}$ copolypeptides in high purity and overall good yields (see SI Table S1).[30]

Table 1. Properties of $\mathbf{M^{O}_{x}A^{DH}_{y}}$ block copolypeptides.

Sample	Composition[a]	*Đ* [b]	Diameter (nm)[c]	PDI[d]
$\mathbf{M^{O}_{35}A^{DH}_{30}}$	$\mathbf{M^{O}_{35}A^{DH}_{30}}$	1.15	251	.354
$\mathbf{M^{O}_{60}A^{DH}_{30}}$	$\mathbf{M^{O}_{60}A^{DH}_{32}}$	1.41	305	.142
$\mathbf{M^{O}_{60}A^{DH}_{40}}$	$\mathbf{M^{O}_{60}A^{DH}_{42}}$	1.25	644	.358
$\mathbf{M^{O}_{60}A^{DH}_{60}}$	$\mathbf{M^{O}_{60}A^{DH}_{63}}$	1.33	567	.302
$\mathbf{M^{O}_{60}A^{DH}_{100}}$	$\mathbf{M^{O}_{60}A^{DH}_{99}}$	1.32	275	.221

[a] Copolypeptide compositions determined by ^{1}H NMR. [b] Dispersity ($Đ = M_w/M_n$) of copolypeptides determined using gel permeation chromatography (GPC). [c] Average hydrodynamic diameter of copolypeptide assemblies determined by DLS measurements on 1 % (w/v) suspensions after extrusion through 1 μm pore size polycarbonate membranes. [d] Polydispersity (PDI) of copolypeptide assemblies determined by DLS.

Compositions and chain lengths of $\mathbf{M^{O}_{x}A^{DH}_{y}}$ copolypeptides were determined using ^{1}H NMR and GPC analysis (Table 1, see SI Table S2 and Figure S2). Since amphiphilic $\mathbf{M^{O}_{x}A^{DH}_{y}}$ samples were not fully soluble in common GPC solvents, these copolypeptides were converted into poly(S-methyl-L-methionine sulfonium chloride)-*b*-poly(dehydroalanine), $\mathbf{M^{M}_{x}A^{DH}_{y}}$, derivatives for GPC analysis in HFIP. All $\mathbf{M^{M}_{x}A^{DH}_{y}}$ samples, as well as $\mathbf{M^{M}}$ precursors, gave monomodal peaks and reasonable dispersity values (Table 1, see SI Table S2 and Figure S2). Absolute chain lengths from end-group analysis by ^{1}H NMR were used for sample labeling since molecular weights from GPC were relative to PEG standards. In order to evaluate how segment length affects aqueous self-assembled structures, $\mathbf{M^{O}_{x}A^{DH}_{y}}$ samples were prepared with a range

of compositions. Initially, a series of samples were prepared with a constant $\mathbf{M^{O}_{x}}$ segment where x = 60, and $\mathbf{A^{DH}_{y}}$ segments where y = 30, 40, 60 and 100 (Table 1). $\mathbf{M^{O}_{60}}$ was chosen since we had previously determined that this length was optimal for vesicle formation in amphiphilic copolypeptides containing α-helical hydrophobic segments, e.g. $\mathbf{M^{O}_{60}(L/F)_{20}}$, **L/F** = poly(L-leucine-*s*-L-phenylalanine).[26] The $\mathbf{A^{DH}_{y}}$ segment lengths were chosen to be greater than 20 residues due to the lower hydrophobicity of dehydroalanine compared to amino acids such as leucine and phenylalanine. A broad range of $\mathbf{A^{DH}_{y}}$ lengths was studied since it was uncertain how the unique chain conformation of $\mathbf{A^{DH}}$ would influence self-assembly.[30]

Assemblies of $\mathbf{M^{O}_{60}A^{DH}_{y}}$ in water were prepared by nanoprecipitation where copolypeptides were first dispersed in DMSO using mild bath sonication, followed by dropwise addition of an equal volume of deionized (DI) water. With vortexing, an additional volume of DI water was added, followed by exhaustive dialysis against DI water to give 1.0% (w/v) $\mathbf{M^{O}_{60}A^{DH}_{y}}$ suspensions. Examination of the suspensions using differential interference contrast (DIC) optical microscopy revealed the presence of round assemblies with sub-micron diameters for all $\mathbf{M^{O}_{60}A^{DH}_{y}}$ compositions (see figure S3). To determine if the assemblies were vesicles or solid particles, the $\mathbf{M^{O}_{60}A^{DH}_{60}}$ assemblies were further examined using laser scanning confocal microscopy (LSCM). Hydrophilic and hydrophobic fluorescent probes were used to distinguish between assembled copolypeptide and encapsulated aqueous media.

Specifically, a suspension of $\mathbf{M^{O}_{60}A^{DH}_{60}}$ was prepared using water containing hydrophilic Texas Red labeled dextran (TR dextran, MW = 3000 Da), where all non-encapsulated TR dextran was subsequently removed from the suspension via dialysis. The copolypeptide assemblies were then labeled by addition of a small volume of hydrophobic $DiOC_{18}$ probe in DMSO.[25] After incubation for 3 hours, the suspension was dialyzed to remove DMSO and excess $DiOC_{18}$. Imaging of these dual labeled suspensions revealed that the $\mathbf{M^{O}_{60}A^{DH}_{60}}$ assemblies are unilamellar water filled vesicles as evidenced by observation of $DiOC_{18}$ labeled polypeptide membranes that

encapsulate TR dextran solution (see Figure S4). The vesicular assemblies of $\mathbf{M^{O}_{60}A^{DH}_{60}}$ were found to be stable in size in 1x PBS buffer at 4 ºC for 30 days via dynamic light scattering (DLS) analysis (see Figure S5).

The formation of unilamellar $\mathbf{M^{O}_{60}A^{DH}_{60}}$ vesicle assemblies was consistent with properties of other block copolypeptide vesicles, which are also all unilamellar.[24-26] This result may be due to the general stiffness of polypeptide membranes due to H-bonding interactions, as compared to more flexible copolymers.[18,19] What was unusual with the $\mathbf{M^{O}_{60}A^{DH}_{y}}$ suspensions was that all of the compositions prepared (y = 30 to 100) were found to form vesicle assemblies of similar size (see Figure S4). In prior studies on self-assembly of amphiphilic block copolypeptides containing α-helical hydrophobic segments, only a narrow range of hydrophobic segment lengths (*ca*. 20 to 30 residues) were found to form vesicles.[24-26] In these studies, shorter hydrophobic segments (*ca*. 10 residues) were found to give spherical micelles, and longer segments (> 40 residues) gave sheets or disordered aggregates.[24,25] In addition, amphiphilic block copolypeptides containing disordered racemic hydrophobic segments formed only spherical or irregular micelles regardless of composition.[22,23] The hydrophobic $\mathbf{A^{DH}}$ segments in $\mathbf{M^{O}_{60}A^{DH}_{y}}$ appear to possess a degree of rigidity that favors low curvature membrane formation,[30] but also more flexibility compared to α-helical polypeptide segments since they do not assemble into flat sheets at greater lengths. Overall, $\mathbf{M^{O}_{x}A^{DH}_{y}}$ assemblies appear to be more tolerant of compositional adjustment compared to other vesicle forming block copolypeptides. We sought to take advantage of this characteristic in the design of $\mathbf{M^{O}_{x}A^{DH}_{y}}$ vesicles capable of stimulus driven inversion.

For successful vesicle inversion, it is desirable that both copolymer segments are similar in length so that a similar, vesicle favoring hydrophilic/hydrophobic balance is obtained in either state. Also, since inversion of $\mathbf{M^{O}_{x}A^{DH}_{y}}$ would give $\mathbf{M_{x}C^{rR}_{y}}$ (Figure 1), the resulting α-helical hydrophobic **M** segments would need to be reasonably short (*ca*. < 40 residues) to favor vesicle formation.[24,25] Consequently, we chose a composition of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ for development of thiol

responsive invertible vesicles. $\mathbf{M^{O}_{35}A^{DH}_{30}}$ was synthesized, and a 1.0 % (w/v) suspension in DI water was prepared, as described above (Table 1, see Table S1, Figure S2). Similar to the $\mathbf{M^{O}_{60}A^{DH}_{y}}$ samples, $\mathbf{M^{O}_{35}A^{DH}_{30}}$ was found to assemble into round assemblies with sub-micron diameters as determined using DIC microscopy and DLS analysis (see Figure S6). Dual labeling of an aqueous $\mathbf{M^{O}_{35}A^{DH}_{30}}$ suspension using TR dextran and $DiOC_{18}$ as described above allowed more detailed characterization of the assemblies. Imaging of the dual labeled suspension confirmed that $\mathbf{M^{O}_{35}A^{DH}_{30}}$ also forms unilamellar water filled vesicles as evidenced by observation of single $DiOC_{18}$ labeled polypeptide membranes surrounding encapsulated TR dextran solution (Figure 3, see Figure S7). The $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles were found to be stable over time (*vide infra*), and were thus considered suitable for studies on amphiphilic inversion using thiol stimuli.

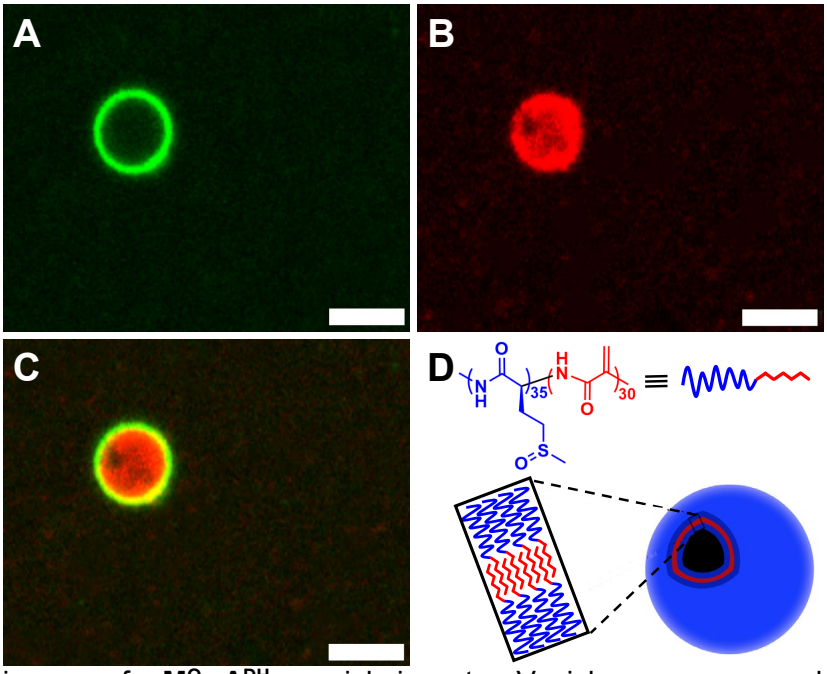

Figure 3. LSCM images of a $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicle in water. Vesicles were prepared in the presence of TR dextran, and hydrophobic domains were labeled by incorporation of $DiOC_{18}$ probe. (A) $DiOC_{18}$ channel. (B) TR dextran channel. (C) Overlay of A and B. (D) Schematic showing amphiphilic nature of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ chains (endgroups not shown), and their proposed assembly into

unilamellar vesicles in water. z direction slice = 0.10 μm. Scale bars = 800 nm. Blue = hydrophilic; red = hydrophobic.

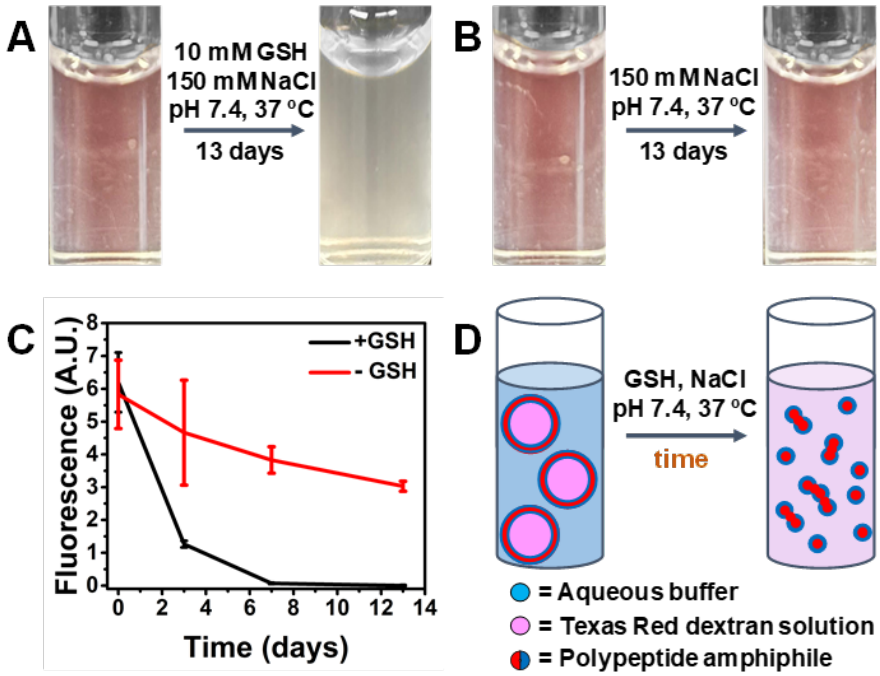

Figure 4. Release of TR dextran cargo from $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles in the presence of glutathione (GSH) under intracellular mimetic conditions. Images of 1.0 % (w/v) suspensions of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles containing encapsulated TR dextran initially and after 13 days (A) with and (B) without addition of glutathione. All images of vials were taken after removal of non-encapsulated TR dextran by dialysis. (C) Graph showing retention of TR dextran in vesicles quantified by measuring fluorescence intensity of dialyzed vesicle suspensions over time (A.U. = arbitrary units). (D) Schematic showing release of TR dextran cargo upon disruption of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles after reaction with glutathione.

We first attempted to invert $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles under conditions mimicking the reducing environment of cell cytosol. Thiol containing glutathione (GSH) is present intracellularly at concentrations ranging from 0.1 to 15 mM,[35] and has been often used as a trigger to release cargos from therapeutic carriers within cells.[4-6] A 1.0 % (w/v) suspension of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles with encapsulated TR dextran cargo was incubated with 10 mM GSH in aqueous 150 mM NaCl

at pH 7.4 and 37 ºC. We expected that GSH would react with both segments in the copolypeptides to reverse amphiphilicity by conversion of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ to poly(L-methionine)$_{35}$-*b*-poly(S-(glutathione)-*rac*-cysteine, Na^+ salt)$_{30}$, $\mathbf{M_{35}C^{rGT}_{30}}$ (see Figure S8). Aliquots of the reaction mixture were taken at different time points, dialyzed to remove non-encapsulated TR dextran, and the fluorescence intensity of remaining encapsulated TR dextran was recorded. After 13 days, all encapsulated TR dextran had been released from $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles in the presence of GSH (Figure 4). In a control study without GSH, greater than 60% of the TR dextran remained encapsulated in the vesicles over the same time period (Figure 4). These results show that $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles possess the ability to release aqueous cargo in response to GSH, which may be useful for therapeutic delivery applications. ^{1}H NMR analysis of copolypeptide isolated from the GSH reaction after 13 days also showed complete modification of $\mathbf{A^{DH}}$ residues with glutathione, and 90% reduction of $\mathbf{M^{O}}$ residues to **M** residues, confirming effective reversal of block copolypeptide amphiphilicity. However, examination of the $\mathbf{M_{35}C^{rGT}_{30}}$ suspension after reaction with GSH using DIC microscopy revealed only the presence of irregular aggregates (see Figure S9). No inverted vesicles were formed, possibly due to limited solubility of $\mathbf{C^{rGT}}$ chains in water.

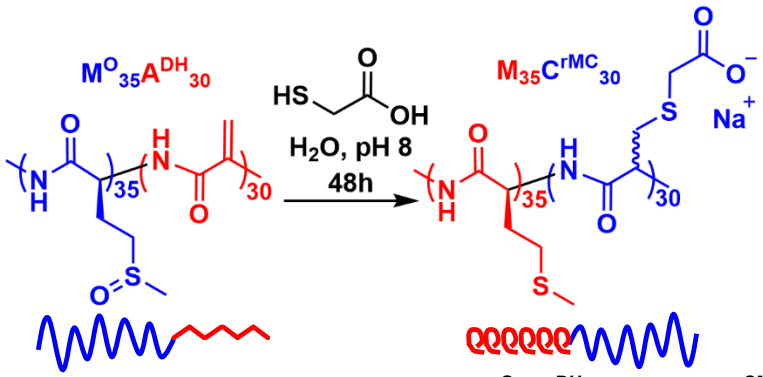

Figure 5. Reversal of amphiphilicity by conversion of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ into $\mathbf{M_{35}C^{rCM}_{30}}$ (endgroups not shown). Blue = hydrophilic; red = hydrophobic.

To improve the likelihood of vesicle inversion, we studied the reaction of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ with a more hydrophilic thiol, thioglycolic acid (TGA), which is known to give water soluble poly(S-carboxymethyl-*rac*-cysteine), $\mathbf{C^{MC}}$, upon reaction with $\mathbf{A^{DH}}$ chains.[30] A 1.0 % (w/v) suspension of

$\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles with encapsulated TR dextran cargo was incubated with excess TGA in DI water at pH 8.0 and 20 ºC. We expected that TGA would reverse amphiphilicity by conversion of $\mathbf{M^{O}_{35}A^{DH}_{30}}$ to poly(L-methionine)$_{35}$-*b*-poly(S-(carboxymethyl)-*rac*-cysteine, Na^+ salt)$_{30}$, $\mathbf{M_{35}C^{rMC}_{30}}$ (Figure 5). After 48 hours, copolypeptide isolated from an aliquot of the suspension was found to have been completely converted from $\mathbf{M^{O}_{35}A^{DH}_{30}}$ to $\mathbf{M_{35}C^{rCM}_{30}}$ by ^{1}H NMR. After dialysis of this suspension, it was analyzed using DIC microscopy and DLS, which showed the presence of round assemblies with sub-micron diameters (see Figure S10). Further analysis of the suspension after $DiOC_{18}$ labeling using LSCM confirmed the formation of inverted unilamellar vesicles containing encapsulated TR dextran (Figure 6, see Figure S11). The amount of encapsulated TR dextran was quantified using fluorescence measurements and was found to be *ca*. 13% of the amount originally present in the $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles. Remarkably, the inverted $\mathbf{M_{35}C^{rCM}_{30}}$ vesicles were able to form *in situ* during the course of amphiphile reversal as TGA reacted with both copolypeptide side chains. It appears that some TR dextran aqueous cargo was also retained or encapsulated during vesicle inversion. To independently verify that $\mathbf{M_{35}C^{rCM}_{30}}$ forms stable vesicles, we prepared and isolated a sample of $\mathbf{M_{35}C^{rCM}_{30}}$ and used the nanoprecipitation protocol described above to prepare a 1.0 % (w/v) $\mathbf{M_{35}C^{rCM}_{30}}$ suspension in DI water, which was found to give vesicles similar to those formed *in situ* (see Figure S12).

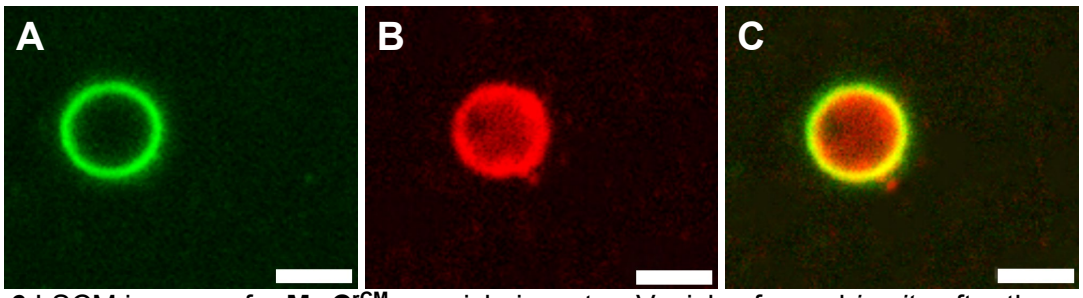

Figure 6 LSCM images of a $\mathbf{M_{35}C^{rCM}_{30}}$ vesicle in water. Vesicles formed *in situ* after the reaction of TR dextran containing $\mathbf{M^{O}_{35}A^{DH}_{30}}$ vesicles with thioglycolic acid. Hydrophobic domains were labeled by incorporation of $DiOC_{18}$ probe before imaging. (A) $DiOC_{18}$ channel. (B) TR dextran channel. (C) Overlay of A and B. z direction slice = 0.10 μm. Scale bars = 700 nm.

Conclusions

A route was developed for preparation of amphiphilic $\mathbf{M^O_xA^{DH}_y}$ diblock copolypeptides, which were found to self-assemble into sub-micron diameter unilamellar vesicles in aqueous media. Formation of vesicles was observed over an unprecedented range of copolypeptide compositions due to the unique properties of $\mathbf{A^{DH}}$ hydrophobic segments, which possess chain conformations that differ greatly from those of α-helical or racemic polypeptides.[30] This expanded compositional freedom is advantageous for design of nanoscale copolypeptide vesicles with adjustable properties. $\mathbf{M^O_{35}A^{DH}_{30}}$ vesicles were used to study reversal of copolypeptide amphiphilicity in response to addition of thiol stimuli. $\mathbf{M^O_{35}A^{DH}_{30}}$ vesicles were incubated with GSH under conditions mimicking an intracellular environment, where both segments of the vesicles were able to fully react with GSH resulting in vesicle disruption and complete release of aqueous cargo. Further, successful triggered inversion of block copolypeptide vesicles in aqueous media under biologically relevant conditions was achieved using $\mathbf{M^O_{35}A^{DH}_{30}}$ vesicles and TGA as a hydrophilic thiol trigger. In these reactions, the different functional groups in each copolypeptide segment were able to react efficiently with a single thiol trigger to reverse copolypeptide amphiphilicity. This ability to convert stable biomimetic vesicles into stable inverted biomimetic vesicles using a mild, biologically relevant stimulus is unprecedented. Since $\mathbf{M^O}$ has been found to be biocompatible and degradable *in vivo*,[20,21] these materials have potential for applications in controlled release of hydrophilic therapeutics and triggered encapsulation of biological media.[25,26]

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.########.

Detailed experimental procedures and data, additional figures, tables, and spectral data (PDF).

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Notes

The authors declare no competing financial interest.

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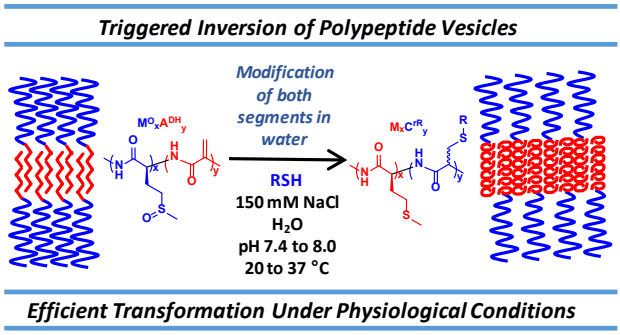
TOC Graphic:


Triggered Inversion of Polypeptide Vesicles
Modification of both segments in water
RSH
150 mM NaCl
H2O
pH 7.4 to 8.0
20 to 37 °C
Efficient Transformation Under Physiological Conditions