## Title

Enzyme-Triggered Cargo Release from Methionine Sulfoxide Containing Copolypeptide Vesicles



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## Authors

Rodriguez, April R
Kramer, Jessica R
Deming, Timothy J

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# Enzyme triggered cargo release from methionine sulfoxide containing copolypeptide vesicles

*April R. Rodriguez,[†] Jessica R. Kramer,[‡] and Timothy J. Deming[†‡*]*

† Department of Bioengineering, University of California, Los Angeles, California 90095, USA.

‡ Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, USA.

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**Abstract**

We have developed a facile, scalable method for preparation of enzyme responsive copolypeptide vesicles that requires no protecting groups or expensive components. We designed amphiphilic copolypeptides containing segments of water soluble methionine sulfoxide, $M^O$, residues that were prepared by synthesis of a fully hydrophobic precursor diblock copolypeptide, poly(L-methionine)$_{65}$-b-poly(L-leucine$_{0.5}$-*stat*-L-phenylalanine$_{0.5}$)$_{20}$, $M_{65}(L_{0.5}/F_{0.5})_{20}$, followed by its direct oxidation in water to give the amphiphilic $M^O$ derivative, $M^O_{65}(L_{0.5}/F_{0.5})_{20}$. Assembly of $M^O_{65}(L_{0.5}/F_{0.5})_{20}$ in water gave vesicles with average diameters of a few microns that could then be extruded to nanoscale diameters. The $M^O$ segments in the vesicles were found to be substrates for reductase enzymes, which regenerated hydrophobic M segments and resulted in a change in supramolecular morphology that caused vesicle disruption and release of cargos.

## Introduction

Stimuli responsive polymeric assemblies provide advantages for many applications, including triggered intracellular release of cargos for drug delivery. Enzyme responsive materials are especially advantageous since they not only utilize the high specificity of enzymes, they can also respond selectively to local biological environments, such as diseased or injured tissues. However, construction of such materials often requires use of complex components and multiple chemical steps. We have developed a facile, scalable method for preparation of enzyme responsive copolypeptide vesicles that requires no protecting groups or expensive components. These materials utilize cell compatible polymers and were found to be excellent substrates for ubiquitous intracellular reductases. This unprecedented enzymatic vesicle reduction caused changes in chain conformations and solubility that resulted in vesicle rupture and release of encapsulated cargos. These fully synthetic materials showcase a new type of enzyme responsive polymer that provides an innovative mechanism for disruption of self assemblies.

Enzyme responsive materials (ERMs) show much promise and are receiving increasing attention since they allow synthetic materials to interface with biological systems, and can utilize the specificity, selectivity, and high catalytic efficiency of enzymes to alter material properties.[1] Numerous ERMs have been developed based on hydrogel or micelle structures, which can be switched between assembled and disassembled forms using enzymes under mild conditions.[1,2] Although many different enzymes and substrates have been used in ERMs, most of these rely on bond formation or cleavage reactions that primarily employ proteases, nucleases, or kinases/phosphatases.[1] In contrast, there are few examples of ERMs that utilize other types of enzymatic reactions, such as oxidations/reductions where only chemical responsive systems have been reported.[3,5] Many ERMs also utilize specific peptide or oligonucleotide sequences as

enzyme substrates,[1,2] which in turn increases the cost and complexity of ERM preparation. To enable widespread use of ERMs in various applications, such as diagnostics, sensors, drug delivery, adaptive surfaces, and regenerative medicine, it would be advantageous to be able to create ERMs that respond to different classes of enzymatic reactions, and that can be prepared using atom economical, scalable methods.

Polymer vesicles are attractive nanocarriers since they have high stability compared to liposomes, and contrary to micelles, are able to encapsulate both hydrophilic and hydrophobic cargos.[6] Although many stimuli responsive polymer vesicles (e.g. to pH, temperature, light) have been prepared, no enzyme responsive vesicles have been reported.[7] Such ERM properties would be useful in adding specific mechanisms for vesicles to interact with cells and biological systems, such as enzyme triggered assembly or disassembly. Our lab has been interested in developing block copolypeptide vesicles since they possess the attractive features of biodegradability, tunable properties, and ability to incorporate the functionality of proteins via a scalable synthetic process.[8,9] We sought to utilize this inherent functionality of polypeptide segments to introduce enzyme responsive properties into vesicles. Although the degradation of synthetic polypeptides by enzymes is well known (e.g. trypsin digestion of poly(L-lysine)),[10] this strategy was not pursued for ERM development since such peptidolysis is essentially a biodegradation process rather than a triggered response.

## 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 solvents with nitrogen prior to use. $^{1}$H 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 and spectra were 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 $10^3$Å Phenomenex Phenogel 5 μm columns using 0.10 M LiBr in DMF as the eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 5 mg/mL. Millipore (18 MΩ) 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.[11] L-Phenylalanine and L-leucine NCAs were synthesized by phosgenation and purified by recrystallization. L-Methionine NCA was prepared by phosgenation and purified by anhydrous column chromatography.[12] α-Methoxy-ω-isocyanoethyl-poly(ethylene glycol)$_{45}$ ($mPEG_{45}$-NCO) was prepared by reacting α-methoxy-ω-aminoethyl-poly(ethylene glycol)$_{45}$ ($mPEG_{45}$-$NH_2$, $M_n$ = 2000 g $mol^{-1}$, Nanocs) with phosgene in THF for 16 h.[12] All block copolypeptides were synthesized using $(PMe_3)_4Co$ initiator.[13]

**Preparation of copolypeptide vesicles** Copolypeptide powder, $M^O_{65}(L_{0.5}/F_{0.5})_{20}$, was dispersed in THF to give a 1 % (w/v) suspension. The suspension was placed in a bath sonicator for 30 minutes to evenly disperse the copolypeptide and reduce large particulates. An equivalent volume of Millipore water was then added to give a 0.5 % (w/v) suspension. The suspension became clear as the sample was mixed by vortex. The mixture was then dialyzed (2,000 MWCO) against Millipore water overnight with three water changes, yielding the copolypeptide vesicle suspension.

**Extrusion of copolypeptide vesicles** Aqueous vesicle suspensions of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ were diluted with Millipore water 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.

**Enzymatic reduction of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles** A 1 % (w/v) suspension of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles was diluted to 0.1 % (w/v) with Millipore water containing 20 mM Tris-HCl, 10 mM $MgCl_2$, 30 mM KCl, 20 mM DTT and 1 μg each of methionine sulfoxide reductase A and methionine sulfoxide reductase B (Prospec-Tany TechnoGene Ltd. Ness Ziona, Israel).[14] A control sample was also prepared as above but without the addition of the enzymes. The suspensions were placed in a 37 °C water bath for 16 h. The suspensions were then imaged using DIC to study effects on vesicle morphology.

**Probe leakage from enzymatically reduced $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles.** A 1 % (w/v) suspension of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles encapsulating Texas Red labeled dextran, was diluted to 0.1 % (w/v) with Millipore water containing 20 mM Tris-HCl, 10 mM $MgCl_2$, 30 mM KCl, 20 mM DTT and 1 μg each of methionine sulfoxide reductase A and methionine sulfoxide reductase B. The diluted suspension (1 mL) was added to an 8000 MWCO dialysis bag and dialyzed against 20 mM Tris-HCl, 10 mM $MgCl_2$, 30 mM KCl, 20 mM DTT (250 mL) at 37 ºC in the dark. A control sample was also prepared as above but without the addition of the enzymes. Aliquots (20 μL) were removed from the dialysis bag at different time points. DMSO (180 μL) was added to each aliquot and the resulting suspensions were sonicated before the excitation

(589 nm) and emission (615 nm) spectra of the Texas Red probe was measured on a QuantaMaster 40 UV Vis spectrofluorometer (Photon Technology International Inc., Birmingham, NJ). Percent dye release over time for both samples was calculated by comparison of measured emission intensities from those of a standard Texas Red dextran solution of identical starting concentration that was kept in identical conditions but not dialyzed. % release = 100 x (emission of standard – emission of sample)/(emission of standard).

**Results and Discussion**

In an effort to create enzyme responsive polypeptide assemblies, we designed amphiphilic copolypeptides containing segments of oxidized methionine residues, which occur naturally when methionine containing proteins are exposed to reactive oxygen species (ROS). Methionine oxidation in proteins is well known and is believed to help maintain protein activity since these residues act as sacrificial substrates for ROS, preventing irreversible oxidation at critical active site residues such as cysteines.[15-17] Methionine residues ,are particularly effective ROS scavengers, able to consume ROS catalytically since their oxidation is reversible via the methionine sulfoxide reductase A and B (MSR) enzymes that are found within cells throughout the human body.[18,19] The two MSR enzymes reduce different diastereomers of methionine sulfoxide that arise from sulfoxide group chirality.[19]

The possibility to interchange methionine residues between hydrophobic (reduced) and hydrophilic (oxidized) states under biological conditions inspired us to incorporate poly(L-methionine), M, segments into copolypeptide vesicle assemblies.[12] Block copolymers containing oxidized M segments have not been previously reported. To evaluate their suitability for use in amphiphilic copolymers, we prepared a poly(L-methionine)$_{80}$, $M_{80}$, homopolymer and subjected it to different levels of chemical oxidation. Mild oxidation of $M_{80}$ using hydrogen peroxide (30

min, 0 ºC) gave poly(L-methionine sulfoxide), $M^{O}_{80}$, while extended oxidation (3 h, 20 ºC) gave the fully oxidized poly(L-methionine sulfone), $M^{O2}_{80}$ (Figure 1a).[20] While M is α-helical and hydrophobic (Figure 1b),[21] $M^{O}$ is known to be highly water soluble and was observed by circular dichroism spectroscopy (CD) to be completely disordered (Figure 1c).[22] Although the sulfone group is more polar than the sulfoxide,[23] the previously unreported, fully oxidized $M^{O2}_{80}$ was observed by CD to be predominantly α-helical (Figure 1d) and only marginally water soluble, likely due to interactions between sulfone groups and crystallization of the helices.[23,24] The water solubility and disordered conformation of $M^{O}$ make it an ideal candidate hydrophilic segment for use in vesicle preparation.[8,9,11] The homopolypeptide $M^{O}$ has also been reported to be non-toxic at 2.0 g/kg when administered intravenously in mice,[25] and we have found that $M^{O}$ is digested by proteinase K, indicating it is also biodegradable (see supporting information, SI).

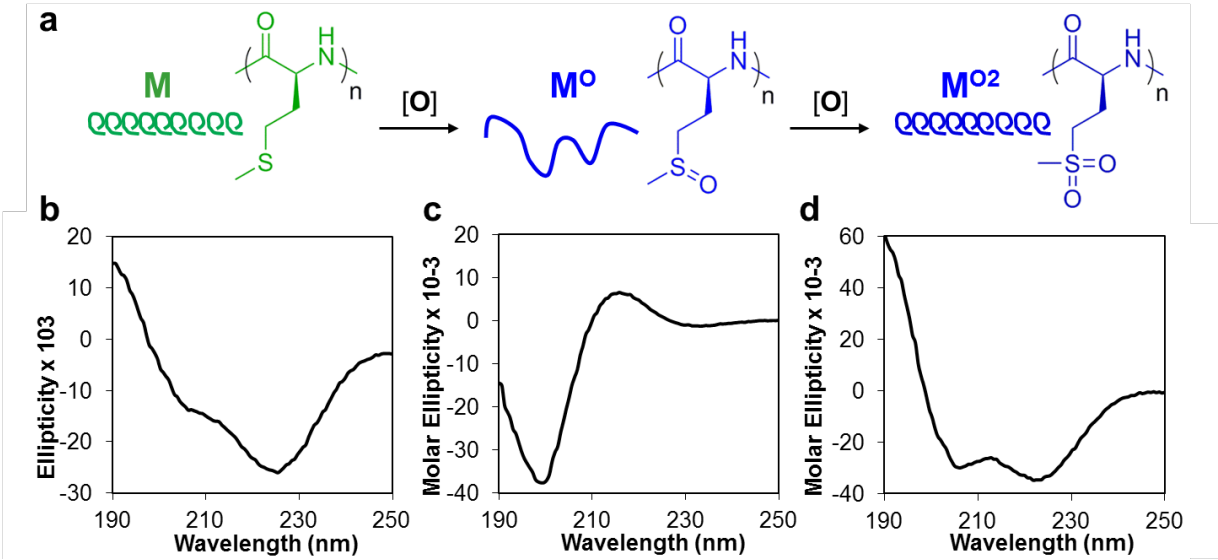


**Figure 1.** Natural and oxidized poly(L-methionine). (a) Structures and schematic drawings of M, $M^{O}$, and $M^{O2}$ homopolymers. Circular dichroism spectra of b) $M_{80}$ prepared as a thin film cast from a 0.25 mg/mL solution in THF, 20 ºC. Ellipticity is reported in degrees×cm$^2$. Since sample

was a solid film, molar ellipticity could not be calculated; c) $M^{O}_{80}$ at 0.25 mg/mL in water, 20 ºC; and d) $M^{O2}_{80}$ at 0.25 mg/mL in water, 20 ºC. [O] = oxidation step.

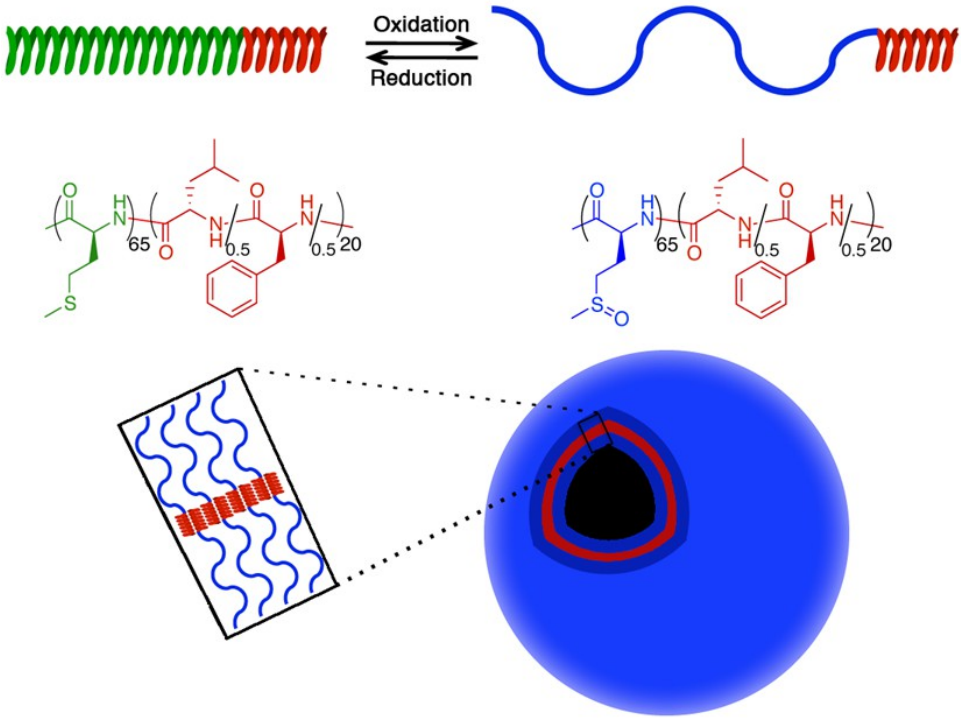


**Figure 2**. Schematic showing structure, redox properties, and proposed self-assembly of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ copolypeptides into vesicles.

In addition to these desirable properties, $M^{O}$ also offers advantages for the synthesis of copolypeptide vesicles in terms of atom economy, cost, and scalability relative to preparation of other peptide containing nanocarriers. 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, $M^{O}$ 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$_{0.5}$)$_{20}$, $M_{65}(L_{0.5}/F_{0.5})_{20}$, was

synthesized via cobalt catalyzed living polymerization of the corresponding *N*-carboxyanhydride monomers (Figure 2, see SI, Table S1).[13] The segment lengths were based on those previously found to promote vesicle formation using other amino acids,[8,9,11] and phenylalanine was incorporated within the rod-like, permanently hydrophobic leucine segment to enhance membrane flexibility.[26] Subsequent mild oxidation of this hydrophobic precursor with hydrogen peroxide directly gave the desired amphiphilic methionine sulfoxide derivative, $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$, in high yield and purity (Figure 2).[20] Due to its use in animal feeds, more methionine is produced chemically than any other amino acid,[27] which lowers its cost and makes this route a very economical process for synthesis of amphiphilic copolypeptides.

**Figure 3.** Images of methionine sulfoxide containing copolypeptide vesicles. (a) DIC image of a 1 % (w/v) $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ aqueous vesicle suspension. (b) LSCM image of a fluorescein labeled

1 % (w/v) $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ aqueous vesicle suspension. (c) TEM image of negatively stained 0.1 % (w/v) $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ aqueous 0.1 μm extruded vesicle suspension. (d) Cryo-TEM image of vitrified 0.1 % (w/v) $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ aqueous 0.1 μm extruded vesicle suspension. Scale bars: a,b = 5 μm; c,d = 200 nm.

Assembly of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ in water using mixed solvent annealing followed by dialysis gave polydisperse vesicles with average diameters of a few microns,[11] as determined by optical microscopy and laser scanning confocal microscopy (LSCM) (Figure 3a,b). These vesicles formed stable suspensions that did not aggregate or precipitate over time in aqueous media, and possessed slightly negative zeta potentials across a broad pH range (see SI, Figure S1). For use in blood circulation where nanoscale diameters are required, the vesicles can be reduced in size by extrusion through polycarbonate filters to give nanoscale vesicles with average diameters of ca. 100 nm as determined by TEM, cryo-TEM and dynamic light scattering (DLS) (Figure 3c,d, see SI, Table S2). Overall, $M^{O}$ was found to serve as an excellent hydrophilic segment for creation of stable, copolypeptide vesicles of controllable size.

Beyond these attractive properties, we also wanted to test if $M^{O}$ segments are able to serve as good substrates for reduction by MSR enzymes. Since conversion of $M^{O}$ to M would result in a conformational change and a decrease in hydrophilicity, successful enzymatic reduction of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles could lead to a triggered change in self-assembled morphology.[2] Oxidation and reduction of carefully positioned methionine residues have been used previously to alter chain conformations of specific peptide and protein sequences, yet these transformations were accomplished only via chemical treatments.[28,29] Although intracellular MSR enzymes are known to efficiently catalyze the thioredoxin dependent reduction of monomeric and protein bound methionine sulfoxide to methionine,[19] to our knowledge the

reduction of methionine sulfoxide residues in synthetic polypeptides using MSR enzymes has not been demonstrated. To assess their ability to act as viable enzyme substrates, we incubated $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles with MSR enzymes and DTT, which was used as a surrogate reductant in place of thioredoxin.[14] Samples were observed to be unchanged when incubated with DTT alone, but the vesicles formed precipitates when incubated with both DTT and MSR enzymes at 37 ºC for 16h (Figure 4a,b,c). Examination of the precipitate from the reduced vesicle sample revealed that irregular sheet-like structures had formed. $^{1}$H NMR analysis of the precipitates confirmed that considerable reduction of $M^{O}$ to M had occurred in the vesicles (see SI, Figure S2), showing that the synthetic $M^{O}$ segments are good substrates for MSR enzymes. Lysine based vesicles, i.e. $K_{60}L_{20}$,[8,9] treated under identical conditions were found to be unaffected, which further supports that precipitation of $M^{O}$ vesicles was due to enzyme reduction (Figure 4d).

**Figure 4.** DIC images of (a) $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicle suspension, (b) $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles incubated with DTT and methionine sulfoxide reductase A and B at 37 ºC for 16 h, (c) $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles incubated with DTT at 37 ºC for 16 h, (d) $K_{60}L_{20}$ vesicles incubated with DTT and methionine sulfoxide reductase A and B at 37 ºC for 16 h. Scale bars = 5 μm.

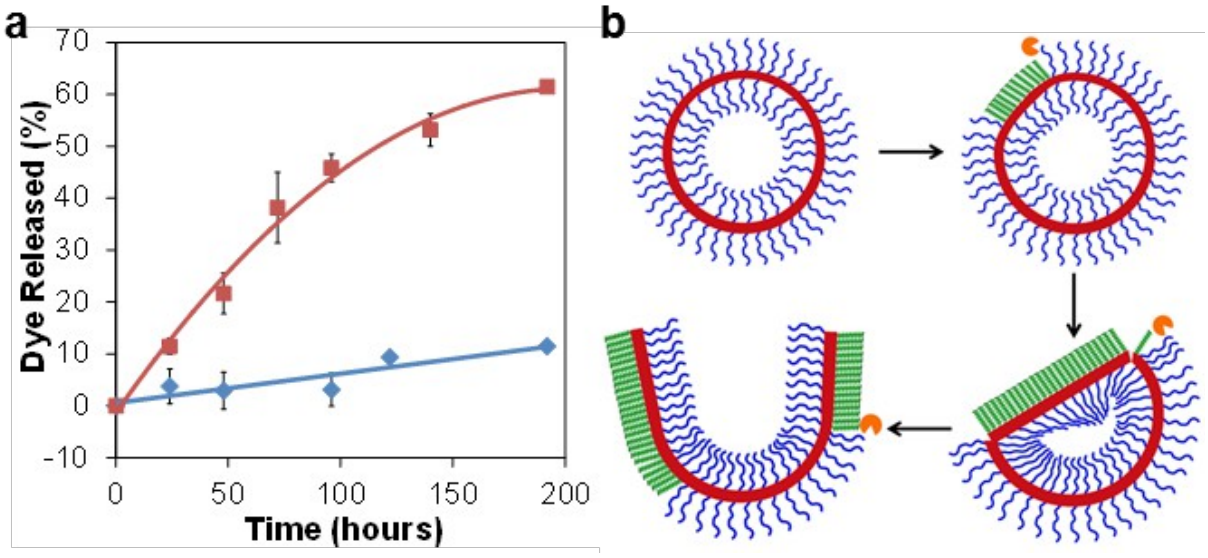


**Figure 5.** Enzyme triggered release of probe molecules from methionine sulfoxide containing vesicles. (a) plot showing cumulative release of Texas Red labeled dextran from $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles over time. Blue diamonds = $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles incubated with DTT at 37 ºC for 16 h; Red squares = $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles incubated with DTT and methionine sulfoxide

reductase A and B at 37 ºC for 16 h. (b) Schematic showing a possible effect of enzymatic reduction of vesicle surface $M^O$ segments to M segments.

The MSR enzyme catalyzed reduction of $M^O$ based vesicles resulted in a subsequent supramolecular change from spherical to a crumpled sheet-like morphology. To determine if this enzymatic response could be used as a trigger to disrupt vesicle membranes and release encapsulated cargos, $M^O_{65}(L_{0.5}/F_{0.5})_{20}$ vesicles containing a model cargo of Texas Red labeled dextran were treated with DTT and MSR enzymes at 37 ºC. Release of the cargo was measured by quantifying the fluorescence remaining within the vesicles over time (Figure 5a). While control vesicles without enzymes released less than 10% of their cargo over 200 hours, enzyme treated vesicles released over 60% of their cargo during the same period. These results confirm that MSR reduction of $M^O$ based vesicles is a viable strategy to trigger release of cargos from these carriers.

Vesicle membrane rupture upon reduction by MSR enzymes is envisioned to occur through transformation of disordered, hydrophilic $M^O$ segments on the vesicle surface to α-helical, hydrophobic methionine rich segments. Aggregation of these rigid, hydrophobic segments should act to stiffen the vesicle membranes, such that vesicle membrane curvature becomes progressively disfavored,[30] eventually causing membrane rupture once a critical level of $M^O$ reduction is reached. A possible representation of this process is visualized in Figure 5b. Since MSR enzymes are normally found within cell cytoplasm, cargo release from the vesicles should only occur intracellularly in a biological setting. If necessary, preparation of $M^O$ vesicles containing additional segments or functionality for increased cell uptake can be accomplished using established procedures.[31,32] The oxidized nature of $M^O$ vesicles should also allow them to remain stable and intact extracellularly, as well as in oxidatively stressed environments, which

are commonly found in injury, cancer and other diseases. Since evidence shows that thioredoxin and MSR enzymes are upregulated by cells in areas of oxidative stress,[33] the selectivity of MSR enzymes for $M^O$ vesicle reduction may also provide a means for targeted cargo release in oxidatively stressed tissues.

## Conclusions

Here we have shown that use of non-toxic $M^O$ segments in copolypeptide vesicle nanocarriers imparts these materials with many advantageous properties, including good solubility, ability to degrade into natural metabolites, and enzyme responsiveness that permits the unprecedented utilization of common intracellular enzymes to promote vesicle disruption and release of encapsulated cargos. These properties make biocompatible and readily prepared $M^O$ an attractive new class of stimulus responsive polymer,[34] which is sensitive to enzymatic reduction and differs from other enzyme responsive materials that typically utilize coupling or cleavage reactions.[1] The structural simplicity of $M^O$, combined with its economical synthesis and exceptional properties, enable its application to a broad range of stimuli responsive synthetic material challenges.

**Supporting Information** Synthetic procedures, vesicle characterization and imaging, enzyme studies, and spectral data. This material is available free of charge via the Internet at http:// pubs.acs.org.

**Corresponding Author** *demingt@seas.ucla.edu Address: Department of Bioengineering, 5121 Engineering 5, HS-SEAS, University of California, Los Angeles, CA 90095 Fax: (+1) 310-794-5956

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**TOC graphic:**