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Permalink
https://escholarship.org/uc/item/6j66c30x

Journal
Macromolecular Bioscience, 15(1)

ISSN
1616-5187

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Publication Date
2015-01-01

DOI
10.1002/mabi.201400348

Peer reviewed
Blending of diblock and triblock copolypeptide amphiphiles yields cell penetrating vesicles with low toxicity

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Keywords: polypeptide, vesicle, copolymer, blends

Abstract

We prepared dual hydrophilic triblock copolypeptides that form both micron and nanometer scale vesicles in aqueous media. The incorporation of terminal homoarginine segments into methionine sulfoxide based vesicles was found to significantly enhance their cellular uptake compared to non-ionic controls. We also demonstrated that diblock and triblock copolypeptides with similar hydrophobic domains were found to mix well and form vesicle populations with uniform compositions. Blending of amphiphiles in vesicle nanocarriers was found to impart these materials with many advantageous properties, including good cellular uptake while
maintaining minimal toxicity, as well as biological responsiveness to promote vesicle disruption and release of encapsulated cargos.

1. Introduction

Polymeric nanocarriers show great promise for controlled intracellular delivery of therapeutics. For successful translation to applications, it is critical these carriers incorporate many levels of functionality, such as cellular uptake and 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 realization of biomimetic multifunctionality in such materials is challenging.[2] Here, we developed nanoscale vesicles with multiple functionalities via physical blending of diblock and triblock copolypeptide amphiphiles. These nanocarriers were prepared from natural amino acids and were optimized to possess a desirable combination of minimal cytotoxicity and good cellular uptake. Since these carriers incorporate hydrophilic poly(L-methionine sulfoxide), M\textsuperscript{60}, segments they also have the potential to release encapsulated cargos upon reduction by enzymes that are present within cells.[3] The ability to add functionality to copolypeptide vesicles by blending of different component amphiphiles 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] We previously reported vesicles composed of poly(L-homoarginine)\textsubscript{60}-block-poly(L-leucine)\textsubscript{20}, \textit{R\textsuperscript{H}60L\textsubscript{20}}, where the polyguanidinium segments served as hydrophilic domains in vesicle formation, and also promoted cellular uptake similar to the widely known cell penetrating properties of the HIV TAT peptide.[5] However, the cationic \textit{R\textsuperscript{H}60L\textsubscript{20}} vesicles are cytotoxic at high
concentrations, and also have limited ability to release their cargos intracellularly.\cite{6} We recently reported the preparation of enzyme responsive copolypeptide vesicles incorporating non-toxic, water soluble M\(^\circ\) segments where enzymatic reduction of M\(^\circ\) residues caused changes in chain conformations and solubility that resulted in vesicle rupture and release of encapsulated cargos.\cite{7} These materials utilized cell compatible and degradable components and were found to be excellent substrates for ubiquitous intracellular reductases, providing a potential means for intracellular cargo release. However, a limitation of these M\(^\circ\) vesicles, i.e. poly(L-methionine sulfoxide)\(_{65}\)-\textit{block}-poly(L-leucine\(_{0.5}\)-\textit{stat}-L-phenylalanine\(_{0.5}\))\(_{20}\), M\(^\circ\)\(_{65}(L_{0.5}/F_{0.5})_{20}\), was that they contained no functionality to promote cellular uptake.

Here, we sought to combine the cellular uptake capability of R\(_{60}\)L\(_{20}\) with the biocompatibility and cargo release capabilities of M\(^\circ\)\(_{65}(L_{0.5}/F_{0.5})_{20}\) to create vesicle nanocarriers possessing all these desirable features. The challenge of this task was being able to obtain an optimal level of polyguanidinium content to achieve good cell uptake without the carriers becoming adversely toxic. In previous work, we designed and prepared dual hydrophilic triblock copolypeptides with a similar goal, where the copolymers contained a polyguanidinium segment to promote cellular uptake, as well as an anionic or non-ionic segment to minimize toxicity.\cite{8} In these designs, only limited success was achieved since anionic segments bound to the polyguanidinium segments prohibiting cellular uptake, and the non-ionic segments were rod-like \(\alpha\)-helices that disfavored vesicle formation. In contrast, the non-ionic hydrophilic homopolypeptide M\(^\circ\) possesses a disordered conformation in water that helps promote vesicle formation.\cite{7}

Using this insight, we designed new triblock copolypeptides containing both oligoguanidinium and M\(^\circ\) hydrophilic segments, where the lengths of the oligoguanidinium segments were varied to adjust vesicle properties, i.e. poly(L-homoarginine)\(_x\)-\textit{block}-poly(L-methionine sulfoxide)\(_{55}\)-
To fine tune and further optimize cellular uptake and minimize cytotoxicity, we also explored preparation of vesicles composed of blends of these triblock copolypeptides with diblock M_{65}(L_{0.5}/F_{0.5})_{20}. Although vesicles composed of blends of different copolypeptide amphiphiles have not been reported, we previously showed that larger copolypeptide amphiphiles could be blended in hydrogel formulations where properties obtained were a combination of those seen in the individual components.\(^{[9]}\) In these studies, an important requirement for successful blending was found to be the use of amphiphiles with similar hydrophobic segment lengths, while mixture of greatly different hydrophilic segments lengths was tolerated.\(^{[9]}\) If different length hydrophobic segments were mixed, the resulting polydispersity within this domain resulted in destabilization of the hydrogel self-assembled structures. We applied this knowledge here to copolypeptide blending experiments designed to yield vesicle assemblies with tunable properties.

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. \(^1\)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 \(\mu\)m 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. L-Phenylalanine, L-leucine and Nε-trifluoroacetyl- L-lysine NCAs were synthesized by phosgenation and purified by recrystallization.\(^{[5,8]}\) L-Methionine NCA was prepared by phosgenation and purified by anhydrous column chromatography.\(^{[10]}\) α-Methoxy-ω-isocyanatoethyl-poly(ethylene glycol)\(_{45}\) (mPEG\(_{45}\)-NCO), used to endcap polypeptide chains to determine their molecular weights (\(M_n\)), 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.\(^{[10]}\) All triblock copolypeptides were synthesized using (PMet\(_3\))\(_4\)Co initiator using established protocols,\(^{[7,11]}\) and \(M^{O_{65}}(L_{0.5}/F_{0.5})_{20}\) was prepared as previously described.\(^{[7]}\)

**Preparation of copolypeptide vesicles.** Copolypeptide powder (\(M^{O_{65}}(L_{0.5}/F_{0.5})_{20}\), \(R^{H}_{10}M^{O_{55}}(L_{0.5}/F_{0.5})_{20}\), \(R^{H}_{20}M^{O_{55}}(L_{0.5}/F_{0.5})_{20}\), or mixtures of these) was dispersed in THF to give a 1 % (w/v) suspension. The suspension was placed in an ultrasonic bath 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 it was mixed by vortex. The mixture was then dialyzed (2,000 MWCO) against Millipore water overnight with three water changes, yielding the copolypeptide vesicle suspensions.

**Extrusion of copolypeptide vesicles.** Aqueous vesicle suspensions composed of \(M^{O_{65}}(L_{0.5}/F_{0.5})_{20}\), \(R^{H}_{10}M^{O_{55}}(L_{0.5}/F_{0.5})_{20}\), \(R^{H}_{20}M^{O_{55}}(L_{0.5}/F_{0.5})_{20}\), or mixtures of these, 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.

**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.

**Cellular uptake of vesicles.** HeLa cells were seeded at a density of 4x10^5 cells/cm^2 and incubated overnight prior to the experiment. The cells were then seeded onto 8-well chambered coverglasses for confocal microscopy experiments or onto 35 mm tissue culture plates for flow cytometry. On the day of the experiment, different fluorescently-labeled extruded vesicles were separately diluted in serum-free media (10 μg/mL for LSCM, 5 μg/mL for flow cytometry) and incubated with HeLa cells for 5 h at 37 ºC to allow the vesicles to be internalized into the cells. Subsequently, the media containing the vesicles was aspirated, and the cells were washed three times with PBS to remove any copolypeptide nonspecifically attached on cell surfaces. Afterwards, the cells were analyzed using either confocal microscopy or flow cytometry to determine the extent of vesicle uptake.

**Laser scanning confocal microscopy (LSCM) of cells.** LSCM images of cells that had been incubated with vesicles were taken on a Leica Inverted TCS-SP MP Spectral Confocal and Multiphoton Microscope (Heidelberg, Germany) equipped with an argon laser (488 nm blue excitation: JDS Uniphase), a diode laser (DPSS; 561 nm yellow-green excitation: Melles Griot), a helium-neon laser (633 nm red excitation), and a two photon laser setup consisting of a Spectra-Physics Millenia X 532 nm green diode pump laser and a Tsunami Ti-Sapphire
picosecond pulsed infrared laser tuned at 768 nm for UV excitation. Note that images were taken using an airy unit of one.

**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.

**Flow cytometry.** Flow cytometric analysis of HeLa cells that had been incubated with 5 µg/mL fluorescein labeled vesicles was performed on a BD FACScan™ (BD Bioscience, San Jose, CA) system equipped with an argon laser (488 nm blue excitation) and two filters: a green filter (530 ± 30 nm) and an orange filter (585 ± 42 nm). A total of 10,000 cells were used for analysis of each sample. All copolypeptide samples were prepared with identical initial fluorescence emission intensities for this experiment. The geometric mean fluorescence intensity was used as a metric to quantify the amount of vesicle internalization into each cell.

**3. Results and Discussion**

To prepare the $R^H_xM^{O}_{55}(L_{0.5}/F_{0.5})_{20}$, $X = 10$ or 20, samples, we first synthesized the corresponding fully hydrophobic precursor triblock copolypeptides, poly($N$,-trifluoroacetyl-L-lysine)$_{20}$-b-poly(L-methionine)$_{20}$-b-poly(L-leucine$_{0.5}$-stat-L-phenylalanine$_{0.5}$)$_{20}$, K$^{TFA}_xM_{55}(L_{0.5}/F_{0.5})_{20}$,
via established protocols using cobalt mediated living polymerization of the corresponding N-carboxyanhydride monomers (Scheme 1, see Supporting Information (SI) Table S1).[7,11] The designed segment lengths and compositions were based on the diblock copolymer \( M^{65}_{L_{0.5}/F_{0.5}} \), which has been shown to readily form stable vesicles in water that can be extruded to nanoscale diameters.[7] Direct oxidation of hydrophobic methionine residues in the new hydrophobic triblock precursors with hydrogen peroxide at 0 ºC gave the corresponding amphiphilic methionine sulfoxide derivatives, \( K_{TFA}M^{55}_{L_{0.5}/F_{0.5}} \), in high yield and purity (Scheme 1, see SI).[12] Using established methods, the lysine residues were then deprotected,[13] followed by guanylation of lysine amine groups[14] to yield the desired copolypeptides \( R^{10}_{10}M^{55}_{L_{0.5}/F_{0.5}} \) and \( R^{20}_{20}M^{55}_{L_{0.5}/F_{0.5}} \), where these short \( R^H \) segments were envisioned to be less cytotoxic than the longer \( R^H_{60} \) segments used in earlier studies.[15]

Similar to \( M^{65}_{L_{0.5}/F_{0.5}} \), assembly of \( R^{10}_{10}M^{55}_{L_{0.5}/F_{0.5}} \) or \( R^{20}_{20}M^{55}_{L_{0.5}/F_{0.5}} \) in water using mixed solvent annealing followed by dialysis gave polydisperse vesicles with average diameters of a few microns,[5,7] as determined by differential interference contrast (DIC) optical microscopy (Figure 1A,B). These vesicles formed stable suspensions that did not aggregate or precipitate over time in aqueous media, and showed that the addition of short \( R^H \) segments in these triblock amphiphiles did not hinder vesicle formation. For use in cell culture studies, the vesicles were reduced in size by extrusion through polycarbonate filters to give stable nanoscale vesicles with average diameters of less than 200 nm as determined by TEM and dynamic light scattering (DLS) (Figure 1C,D, Table 1).

Enabling the ability to fine tune the density of \( R^H \) chains on vesicle surfaces, the triblock copolypeptides were found to readily mix with \( M^{65}_{L_{0.5}/F_{0.5}} \) at any ratio to give stable micron and nanoscale vesicles containing both components (Table 1, see Figure S1). In order to estimate
the degree of diblock and triblock copolymer mixing within the vesicle membranes, we separately labeled each sample with a different fluorescent probe before mixing. Fluorescein was conjugated to thioether groups of methionine side chains in \(M^{O}_{65}(L_{0.5}/F_{0.5})_{20}\) prior to oxidation, and tetramethylrhodamine was conjugated to amino groups of lysine side chains in the triblock copolypeptides. These labeled copolymers were diluted with unlabeled materials to balance fluorescence intensities of the different probes in each mixture. Examination of unextruded, micron size vesicles composed of a mixture of 75 mol% diblock and 25 mol% triblock copolypeptides using laser scanning confocal microscopy (LSCM) showed that both diblock and triblock labeled chains were evenly distributed in all vesicles in expected proportions (Figure 1E,F). Good mixing of the diblock and triblock copolymers within the vesicle membranes was also demonstrated by observation of Förster resonance energy transfer (FRET) from fluorescein probes on the diblock chains to tetramethylrhodamine probes on the triblock chains (see SI Figure S2).[^16]

To assay the ability of RH segments in these diblock/triblock mixed vesicles to promote cellular uptake, HeLa cells were incubated with fluorescein labeled vesicles containing different fractions of triblock copolypeptide. Cell uptake studies revealed that fluorescein labeled \(M^{O}_{65}(L_{0.5}/F_{0.5})_{20}\) vesicles were minimally taken up by HeLa cells (Figure 2A,B), indicating that while the \(M^{O}\) segments provide good biocompatibility,[^17] they are inert toward cell membranes. Increasing the content of either \(RH_{10}M^{O}_{55}(L_{0.5}/F_{0.5})_{20}\) (Figure 2C-F) or \(RH_{20}M^{O}_{55}(L_{0.5}/F_{0.5})_{20}\) (see SI Figure S3) in the mixed composition vesicles gave rise to improved cell uptake, with maximal uptake found in samples containing 50 mol% or greater triblock content. Flow cytometric analysis of cells from these studies also showed that vesicles were taken up by entire cell populations to similar extents, indicating a robust uptake mechanism (Figure 3). Since cellular
uptake did not significantly improve when triblock fractions in the mixed vesicles were greater than 50 mol%, and since increased R\textsuperscript{H} content will likely increase vesicle cytotoxicity (vide infra), we chose the 1:1 mixtures of R\textsuperscript{H}\textsubscript{10}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20} and M\textsubscript{65}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20} as optimized nanovesicle formulations.

Cell viability measurements with HeLa cells exposed to increasing vesicle concentrations of R\textsuperscript{H}\textsubscript{10}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}, R\textsuperscript{H}\textsubscript{20}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}, and their mixtures with M\textsubscript{65}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20} are shown in Figure 4 (see SI Figure S4). Extruded M\textsubscript{65}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20} vesicles were found to be non-toxic to HeLa cells at concentrations up to 100 µg/mL (Figure 4), which confirms that M\textsuperscript{O} serves as an excellent hydrophilic segment for creation of non-toxic, copolypeptide vesicle nanocarriers. While R\textsuperscript{H}\textsubscript{60}L\textsubscript{20} was highly toxic at the lowest concentration studied, pure R\textsuperscript{H}\textsubscript{10}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20} and R\textsuperscript{H}\textsubscript{20}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20} vesicles were found to be less toxic, confirming that shorter R\textsuperscript{H} segments are much better tolerated by the cells. Noteworthy are the optimized vesicles composed of 1:1 mixtures of diblock and triblock copolymers, which showed high cell viabilities similar to pure M\textsubscript{65}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20} and the cell only control (Figure 4). It appears that the non-toxic M\textsuperscript{O} segments present in the M\textsubscript{65}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20} fraction are able to effectively protect cells from damaging effects of R\textsuperscript{H} segments in the triblock chains. Consequently, the blending of these diblock and triblock amphiphiles was found to be a useful method for optimization of cellular uptake and minimization of cytotoxicity.

4. Conclusions

Here we prepared dual hydrophilic triblock copolypeptide vesicles that are able to form both micron and nanometer scale vesicles in aqueous media. The incorporation of terminal homoarginine segments into M\textsuperscript{O} based vesicles was found to significantly enhance their cellular uptake compared to a diblock copolypeptide vesicle control. We also demonstrated that diblock
and triblock copolypeptides with similar hydrophobic domains were found to mix well and form vesicle populations with reasonably uniform compositions. Blending of diblock and triblock copolypeptide amphiphiles in vesicle nanocarriers was found to impart these materials with many advantageous properties, including good cellular uptake with minimal toxicity, as well as capability for biological responsiveness to promote vesicle disruption and release of encapsulated cargos. Hence, the blending of different amphiphiles is a promising means to introduce combinations of desirable functionalities in nanocarrier formulations without requiring the use of complicated synthesis procedures.

**Supporting Information** Synthetic procedures, additional vesicle characterization and imaging, FRET studies, and spectral data. Supporting Information is available from the Wiley Online Library or from the author.

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**Acknowledgement** This work was supported by a grant from the National Science Foundation (DMR 1308081) and by the Department of Defense Prostate Cancer Research Program under award number W81XWH-09-1-0584. Flow cytometry was performed at the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institutes of Health awards CA-16042 and AI-28697, and by the JCCC, the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA.
References


Table 1 Dynamic light scattering (DLS) data on block copolypeptide assemblies. Data are z-average diameters and polydispersities (PDI) of 0.2 % (w/v) vesicle suspensions that were serial extruded through 1.0, 0.4, 0.2, and 0.1 μm PC filters.

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<th>Vesicle Sample</th>
<th>Diameter (nm)</th>
<th>PDI</th>
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<td>0.203</td>
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<td>105</td>
<td>0.200</td>
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<td>75% M_{65}^{0}(L_{0.5}/F_{0.5})_{20}</td>
<td>165</td>
<td>0.197</td>
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<tr>
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<td>160</td>
<td>0.164</td>
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<tr>
<td>50% M_{65}^{0}(L_{0.5}/F_{0.5})_{20}</td>
<td>127</td>
<td>0.177</td>
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<tr>
<td>R_{10}^{H}M_{55}^{0}(L_{0.5}/F_{0.5})_{20}</td>
<td>124</td>
<td>0.162</td>
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<tr>
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<td>0.172</td>
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<td>0.172</td>
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<td>0.212</td>
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Scheme 1  Schematic showing structure, chemical functionalization, and proposed self-assembly of $R^{10}M^{155}(L_{0.5}/F_{0.5})_{20}$ ($x = 10, 20$) triblock copolypeptides into vesicles.
Figure 1 Images of vesicles containing triblock copolypeptides. (A,B) DIC images of 1 % (w/v) vesicle suspensions composed of triblock copolypeptides. (A) $R^{H}_{10}M^{O}_{55}(L_{0.5}/F_{0.5})_{20}$, (B) $R^{H}_{20}M^{O}_{55}(L_{0.5}/F_{0.5})_{20}$. (C,D) TEM images of negatively stained 0.1 % (w/v) triblock copolymer vesicle suspensions that had been serially extruded through 1.0, 0.4, 0.2, and 0.1 µm PC filters. (C) $R^{H}_{10}M^{O}_{55}(L_{0.5}/F_{0.5})_{20}$, (D) $R^{H}_{20}M^{O}_{55}(L_{0.5}/F_{0.5})_{20}$. (E,F) LSCM images of 1 % (w/v) vesicles consisting of a mixture of 25 mol% tetramethylrhodamine labeled $R^{H}_{20}M^{O}_{55}(L_{0.5}/F_{0.5})_{20}$ and 75 mol% fluorescein labeled $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$. (E) Image of fluorescein channel, (F) image of tetramethylrhodamine channel. Scale bars: C,D = 200 nm; A,B,E,F = 5 µm.
Figure 2  LSCM images showing uptake of vesicle suspensions in HeLa cells. Cells were incubated with fluorescein labeled vesicle suspensions composed of 0 to 100 mol% $\mathrm{R}^{10}\mathrm{M}^{55}(\mathrm{L}_{0.5}/\mathrm{F}_{0.5})_{20}$ mixed with $\mathrm{M}^{65}(\mathrm{L}_{0.5}/\mathrm{F}_{0.5})_{20}$. LSCM images of cells incubated for 5 h with (A) no vesicles, cell only control, (B) 0 mol%, (C) 25 %, (D) 50 mol%, (E) 75 mol% and (F) 100 mol% triblock in mixed vesicles. Samples with identical overall copolypeptide concentration (10 $\mu\text{g/mL}$) and fluorescence emission intensity were used for B-F. Scale bars = 20 $\mu\text{m}$. 
Figure 3  Flow cytometry analysis showing populations of HeLa cells that became fluorescently labeled after incubation with 5 µg/mL fluorescein labeled, extruded copolypeptide vesicles. (A) Raw FACS data for each condition. Green = cells only. Red = cells incubated with fluorescein labeled R\textsubscript{H}60L\textsubscript{20} vesicles. Blue = cells incubated with fluorescein labeled vesicles composed of 50 mol% R\textsubscript{H}10M\textsubscript{O}55(L_{0.5}/F_{0.5})\textsubscript{20} mixed with M\textsubscript{O}65(L_{0.5}/F_{0.5})\textsubscript{20}. Orange = cells incubated with fluorescein labeled vesicles composed of 50 mol% R\textsubscript{H}20M\textsubscript{O}55(L_{0.5}/F_{0.5})\textsubscript{20} mixed with M\textsubscript{O}65(L_{0.5}/F_{0.5})\textsubscript{20}. (B) Fluorescence intensities of cell populations relative to the cell only control. Relative Fluorescence = fold increase in geometric mean fluorescence intensity compared to cells only (arbitrary units). R\textsubscript{10} Blend = 50 mol% R\textsubscript{H}10M\textsubscript{O}55(L_{0.5}/F_{0.5})\textsubscript{20} mixed with M\textsubscript{O}65(L_{0.5}/F_{0.5})\textsubscript{20}. R\textsubscript{20} Blend = 50 mol% R\textsubscript{H}20M\textsubscript{O}55(L_{0.5}/F_{0.5})\textsubscript{20} mixed with M\textsubscript{O}65(L_{0.5}/F_{0.5})\textsubscript{20}. Error bars represent the standard deviation from an average of three measurements.
Figure 4  Survival of HeLa cells after incubation with different extruded 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. Green bars = M\textsubscript{65}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}, Red bars = 50 mol\% R\textsuperscript{10}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}:50 mol\% M\textsubscript{65}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}, Blue bars = 50 mol\% R\textsuperscript{10}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}:50 mol\% M\textsubscript{65}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}, Orange bars = R\textsuperscript{10}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}, Violet bars = R\textsuperscript{10}M\textsubscript{55}(L\textsubscript{0.5}/F\textsubscript{0.5})\textsubscript{20}, and Yellow bars = R\textsuperscript{60}L\textsubscript{20}. Error bars represent the standard deviation from an average of three measurements.
Diblock and triblock copolypeptides were blended to form vesicle populations with uniform compositions. Mixing of cationic and non-ionic amphiphiles in these vesicles gave optimized properties of good cellular uptake while maintaining minimal cytotoxicity. Copolypeptide mixing is a promising strategy to provide useful functionality without requiring the use of complicated synthesis procedures.