

Controlling nanoemulsion surface chemistry with poly(2-oxazoline) amphiphiles

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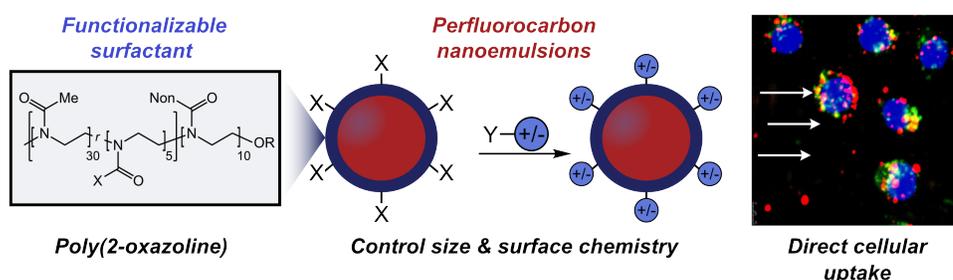
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ABSTRACT: Emulsions are dynamic materials that have been extensively employed within pharmaceutical, food and cosmetics industries. However, their use beyond conventional applications has been hindered by difficulties in surface functionalization, and an inability to selectively control physicochemical properties. Here, we employ custom poly(2-oxazoline) block copolymers to overcome these limitations. We demonstrate that poly(2-oxazoline) copolymers can

effectively stabilize nanoscale droplets of hydrocarbon and perfluorocarbon in water. The controlled living polymerization of poly(2-oxazoline)s allows for the incorporation of chemical handles into the surfactants such that covalent modification of the emulsion

surfaces can be performed. Through post-emulsion modification of these new surfactants, we are able to access nanoemulsions with modified surface chemistries, yet consistent sizes. By decoupling size and surface charge, we explore structure-activity relationships involving the cellular uptake of nanoemulsions in both macrophage and non-macrophage cell lines. We conclude that the cellular uptake and cytotoxicity of poly(2-oxazoline)-stabilized droplets can be systematically tuned via chemical modification of emulsion surfaces.



INTRODUCTION:

Facile methods to reliably prepare complex materials facilitate new technologies and medicines. Towards the development of optoelectronic materials and sensors, predictable assemblies of hard nanomaterials have enabled emergent optical, electronic, and magnetic properties.¹⁻⁴ For biomedical applications, the advantageous safety and clearance properties of soft organic materials have propelled liposomes, polymer micelles, hydrogels, and dendrimers into the research spotlight.⁵⁻¹⁰ Surprisingly, far less attention has been placed on incorporating chemical complexity into emulsions^{11,12} despite their simple formation and ability to encapsulate significant amounts of cargo.¹³⁻¹⁵

Emulsions are liquid-in-liquid droplets stabilized by surfactant, with size distributions ranging from several nanometers to hundreds of micrometers.¹⁶ These materials have traditionally been employed as delivery systems^{17,18} in cosmetic¹⁹, food^{20,21}, and pharmaceutical industries,²²⁻²⁴ with more advanced applications including templates for material synthesis²⁵⁻²⁹ and nanoscale reactors³⁰⁻³³. While these emerging applications showcase the potential versatility of emulsions, liquid droplets remain

underdeveloped compared to other soft materials.^{11,12,34} Currently, a challenge in the preparation of emulsions is decoupling the size and surface charge of the materials.^{35,36} Furthermore, chemically robust approaches to append functionality to the surface are limited.^{12,34,37,38}

Surfactants play a critical role in the formation and stabilization of emulsions, directly affecting the size, surface charge, and stability of the droplets (Figure 1A).^{39,40} This class of amphiphilic molecules can be composed of small molecules or polymers. They orient at the liquid-liquid interface to reduce interfacial tension between the immiscible emulsion core and bulk phases. Simple surfactants such as phospholipids and poloxamers (Figure 1B) are routinely used for industrial applications, while recently engineered peptide⁴¹⁻⁴³, polymer,⁴⁴ and nanoparticle⁴⁵ surfactants have produced responsive materials and sophisticated architectures.^{46,47} Slight changes in surfactant structure can drastically affect the physicochemical properties of the emulsions.³⁸ These subtleties make the systematic alteration of a single characteristic difficult, precluding structure-property relationships. A method that will facilitate the decoupling of size and surface charge is the ability to control surface chemistry after the droplet has been formed.

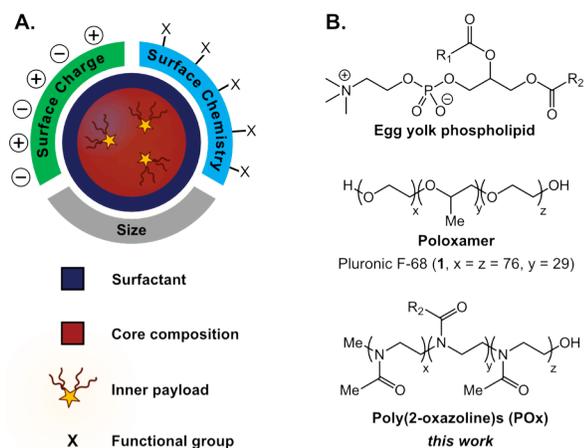


Figure 1. (A) Surfactants dictate the size, charge, and surface chemistry of emulsions. Emulsion cores can be composed of several liquid phases (e.g. oil, perfluorocarbons). Payload can be solubilized in the emulsion core. Functional groups can be appended on the surface. (B) Selected surfactants for emulsion formation, including advantages of poly(2-oxazoline) surfactants presented herein.

Conventional emulsion surface functionalization techniques involve modification of the surfactant prior to emulsification.^{48–50} The functionalized surfactant can be employed solely or in combination with other surfactants. Limitations to this approach are the reliance on cosurfactants⁵¹ and the inability to decouple size and surface charge. An alternative to this strategy is the introduction of a functionalized amphiphile after emulsification that can absorb to the surface.⁴⁸ This competitive absorption mechanism allows for surface chemistry alteration,⁵² but risks desorption of the modified surfactant.³⁴ Other post-emulsification strategies rely on reversible chemical interactions with the surfactant at the liquid-liquid interface. Reported approaches involve electrostatic deposition,^{53–55} designer peptide amphiphiles,^{41,56,57} or reactive copolymer surfactants for disulfide exchange.⁵⁸ These techniques are all environment-dependent, limiting their generalizability. We envisioned that a versatile and robust route toward emulsion surface functionalization would be the use of custom surfactants with chemical handles that undergo covalent modification at the liquid-liquid interface.

When considering the design of surfactants that will enable post-emulsion modification, we looked to amphiphilic copolymers. Polymer surfactants benefit from steric stabilization and tunable properties via alterations in block structure.^{59,60} We hypothesized that chemical handles could be incorporated into the hydrophilic block without destabilizing the droplets. A popular class of polymer surfactants are poloxamers: block copolymers of poly(ethylene oxide) and poly(propylene oxide) (Figure 1B). While poloxamers (e.g. Pluronic F-68, **1**) have been extensively validated as surfactants for emulsions, it is

synthetically challenging to incorporate chemical functionality into them. Additionally, poly(ethylene oxide)-containing amphiphiles, like many surfactants employed for pharmaceutical emulsion formulations,^{61–63} are associated with immunogenicity, making them a poor choice for materials with biomedical applications.^{64,65} Recent work has suggested that poly(2-methyl-2-oxazoline) has similar protein repellent features to poly(ethylene oxide).^{66–68} Additionally, poly(2-oxazoline)s (POx) are synthesized through a controlled, living polymerization, facilitating tunable block structure, length, and selective comonomer addition.^{69,70} Based on these collective attributes, we focused on amphiphilic poly(2-oxazoline) surfactants to decouple the physiochemical properties of nanoemulsions and facilitate control their surface chemistry.

Herein, we report a panel of POx surfactants for the stabilization and functionalization of nanoemulsions, kinetically stabilized emulsions less than 500 nm in size.⁷¹ We showcase the controlled living polymerization of POx to incorporate comonomers into the hydrophilic block of the surfactants to facilitate post-emulsion functionalization. We find that thiol-ene and copper-catalyzed azide-alkyne cycloaddition (CuAAC) chemistries are successful at the liquid-liquid interface, overcoming a key obstacle in emulsion functionalization.^{11,12} We demonstrate that these chemistries can decouple emulsion properties by altering the charge of similarly-sized droplets.

A scenario where both the size and charge of particles directly influence function is cellular uptake. Controlling cell-nanoparticle interactions is essential for advancements in nanomedicine. Previous work has explored the relationship between size, charge, and cellular uptake by modulating the surface chemistry of gold nanoparticles,^{72–74} micelles,⁷⁵ and peptide-brush polymers.⁷⁶ These studies indicate that not only is the surface chemistry important but also the nanomaterial composition. The custom surfactants reported herein allowed us to extend the scope of cellular uptake studies to include nanoemulsions. We find that macrophage and non-macrophage cells display charge-dependent cellular uptake with cationic emulsions preferentially taken up in both cell types.

RESULTS AND DISCUSSION:

We first synthesized a small library of amphiphilic diblock and triblock poly(2-oxazoline) amphiphiles and explored their ability to stabilize oil-in-water and perfluorocarbon-in-water nanoemulsions (Figure 2). The polymer surfactants were designed to mimic Pluronic F-68 with poly(2-methyl-2-oxazoline) replacing poly(ethylene oxide) and either poly(2-propyl-2-oxazoline), poly(2-nonyl-2-oxazoline), or poly(2-(perfluorohexyl)ethyl-2-oxazoline) replacing the hydrophobic poly(propylene oxide). The POx surfactants were synthesized through a controlled, living cationic ring-opening polymerization of corresponding 2-

substituted-2-oxazoline monomers (Figure 2A, 2-5). Due to the controlled nature of the polymerization mechanism, block lengths were tuned by initiator to monomer ratio. Hydrophilic blocks were kept at 30 repeat units of 2-methyl-2-oxazoline (2), while hydrophobic blocks (composed of 3, 4 or 5) were kept at 10 repeat units. Microwave-assisted polymerization allowed for short reaction times and low dispersities (Table 1) compared to traditional solution phase synthesis.⁷⁷ Polymerizations were quenched with acrylic acid to aid determination of number average molecular weight (M_n) via end-group analysis. Following this procedure, we prepared polymers 6-11 (Figure 2A, Table 1) and characterized them to have M_n from 3.7 to 10.1 kDa with narrow dispersities ($\mathcal{D} \leq 1.30$).

Next, we evaluated the ability of poly(2-oxazoline)s 6-11 to act as surfactants for nanoemulsions. Poly(2-oxazoline)s have been thoroughly investigated for micellization⁷⁸ and have previously been employed for chloroform-in-water macroemulsions,⁷⁹ yet their use as surfactants for nanoemulsion formation is novel. Our main interest lies in

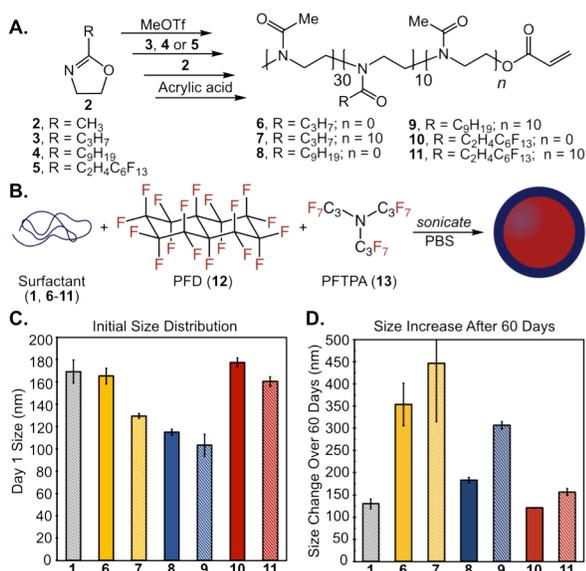


Figure 2. (A) Library of amphiphilic di- and triblock copolymers and (B) their utility as surfactants for PFC nanoemulsions composed of 7:3 v/v% perfluorodecalin (PFD):perfluorotripropylamine (PFTPA). (C) Initial size distributions of POx-stabilized emulsions. Emulsions were prepared by sonicating a solution of 2.8 wt% surfactant, with 10 vol% 7:3 PFD:PFTPA in phosphate buffered saline (PBS). Emulsions were diluted 1:100 in MilliQ water prior to measurements by dynamic light scattering (DLS). (D) Ostwald ripening of emulsions over 60 days. The emulsions prepared in (C) were monitored over time by DLS. (C/D) Data represents the average of 3 independent samples. (C) Error bars represent the half-width at half-maximum averaged over the 3 independent samples. (D) Error bars represent the standard deviation of the size changes for 3 independent samples.

perfluorocarbon-in-water nanoemulsions,⁸⁰⁻⁸² as the orthogonality of the fluoruous phase provides opportunities to selectively sequester fluoruous-tagged payloads inside the droplets.⁸³ Historically, perfluorocarbon (PFC) nanoemulsions have been stabilized by Pluronic F-68 (1) for use as artificial blood substitutes,⁸⁴ however, these surfactants have been associated with formulation inconsistencies and multidose toxicity.⁸⁵⁻⁸⁷ Contemporary applications of PFC nanoemulsions such as ¹⁹F-magnetic resonance imaging,^{88,89} ultrasound contrast agents,^{44,90} photodynamic therapy,^{82,91} and intracellular sensors⁹² have spawned interest in new formulations. We previously looked to commercially available polymers and biomolecules for the stabilization and surface functionalization of PFC emulsions, but found these materials unsuitable due to large size and rapid degradation of the droplets, as well as limitations in post-emulsion functionalization.⁸⁰ Recently, volatile perfluorocarbon droplets have been effectively stabilized by Gianneschi and coworkers through triblock poly(norbornene)s,⁴⁴ and by Medina *et al.* through crosslinked peptides⁴³. However, neither of these efforts explored surface modification. Collectively, these works indicate that custom polymer surfactants allowing for functionalizable, stable PFC nanoemulsions are necessary.

To test the ability for POx amphiphiles to stabilize nanoemulsions, polymers 6-11 were first solubilized in dimethylformamide and then diluted with phosphate buffered saline (PBS, pH 7.4) to a final surfactant loading of 2.8 wt%. This solution was combined with 10 vol% fluoruous or hydrocarbon oil. Emulsions were formulated through ultrasonication for 15 minutes at 0 °C. For the fluoruous solvent, we selected a 7:3 (v/v%) mixture of perfluorodecalin:perfluorotripropylamine (PFD:PFTPA,

Table 1. Surfactant library of amphiphilic poly(2-oxazoline)s

#	Polymer	M_n^a (kDa)	\mathcal{D}
6	P(MeOx ₃₀ - <i>b</i> -PrOx ₈)	3.5	1.2 6
7	P(MeOx ₃₀ - <i>b</i> -PrOx ₉ - <i>b</i> -MeOx ₃₀)	6.2	1.2 9
8	P(MeOx ₃₀ - <i>b</i> -NonOx ₁₁)	4.8	1.2 4
9	P(MeOx ₃₀ - <i>b</i> -NonOx ₁₁ - <i>b</i> -MeOx ₃₀)	7.4	1.2 9
10	P(MeOx ₂₇ - <i>b</i> -FOx ₁₀)	6.6	1.1 6
11	P(MeOx ₃₀ - <i>b</i> -FOx ₉ - <i>b</i> -MeOx ₃₀)	8.9	1.0 9

MeOx: methyl-2-oxazoline, 2; PrOx: propyl-2-oxazoline, 3; NonOx: nonyl-2-oxazoline, 4; FOx: (perfluorohexyl)ethyl-2-oxazoline, 5

^aNumber-average molecular weight (M_n) determined by ¹H-NMR end-group analysis of terminal CH₃ group to polymeric backbone

^bDispersity index (\mathcal{D}) determined by GPC analysis (eluent: DMF + 0.1M LiBr)

12:13) due to its use in Fluosol-DA, a previously FDA-approved PFC nanoemulsion stabilized by Pluronic F-68 (**1**).⁸⁴ Dynamic light scattering analysis of POx-stabilized PFC nanoemulsions showed size distributions that were comparable or smaller than droplets stabilized by **1** (Figure 2C), with polydispersities ranging from 0.1 to 0.2. Monitoring the size over 60 days at ambient temperature indicated that propyl-2-oxazoline-containing surfactants (**6**, **7**) were inferior, exhibiting significant Ostwald ripening (>350 nm change in size, Figure 2D), despite structural analogy to **1**. The more hydrophobic nonyl-2-oxazoline-containing surfactants (**8**, **9**) were superior to the propyl-containing surfactants with diblock **8** performing better than triblock **9**. Surfactants with fluororous components (**10**, **11**) displayed the best stability over time, on par with **1**. When employed for olive oil emulsion formation, **6-9** resulted in sub-250 nm droplets. As expected, the fluororous copolymers **10** and **11** were not effective surfactants for the formation of oil-in-water nanoemulsions. After 3 weeks, oil droplets stabilized by propyl-2-oxazoline-containing surfactants (**6**, **7**) underwent phase separation. In contrast, emulsions stabilized by nonyl-2-oxazoline-containing surfactants (**8**, **9**) showed no size change. These data demonstrate that the following results on PFC nanoemulsions can be extended to more conventional oil-in-water nanoemulsions. Our initial library of poly(2-oxazoline) surfactants resulted in amphiphilic copolymers that performed similarly to Pluronic F-68 yet could be prepared through a controlled living polymerization. Diblock copolymers **8** and **10** stood out as most promising, as **8** formed emulsions of small size for both oil-in-water and PFC nanoemulsions, while **10** formed PFC emulsions with good stability. Work toward expanding this library to elucidate the role of the surfactant's hydrophilic-lipophilic balance, block length and structure on emulsion size and stability are underway. Here, we focus on the creation of POx surfactants that allow for modification of the emulsion surface, such that size and surface charge can be decoupled.

Surfactants that could be further functionalized were synthesized by incorporating alkene and alkyne functionality into the hydrophilic poly(2-methyl-2-oxazoline) block of POx amphiphile **8**. Alkene and alkyne functionalities were chosen due to their ability to undergo thiol-ene and CuAAC “click” chemistries, respectively. These classes of reactions benefit from their high efficiency, modularity, and water compatibility—all desirable characteristics for the proposed post-emulsion modification route.^{93,94} To prepare functionalizable POx surfactants, we initiated the polymerization of 5:30 2-(3-butenyl)-2-oxazoline (**14**) or 2-(4-pentynyl)-2-oxazoline (**15**) to methyl-2-oxazoline (**2**, 15 mol% alkene/alkyne) with methyl triflate. Once all monomer was consumed, nonyl-2-oxazoline (**4**) was introduced to the reaction

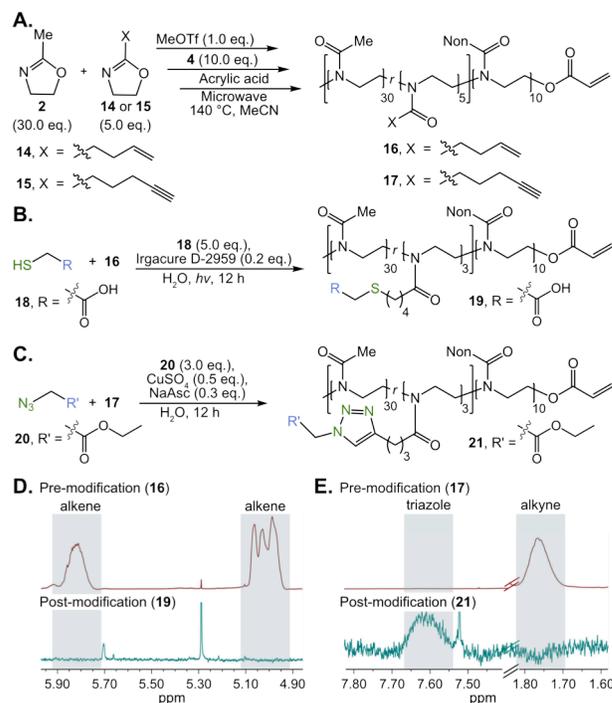


Figure 3. (A) Synthesis of a functionalized POx surfactant. A functional comonomer is randomly incorporated within the hydrophilic block to yield alkene (**16**) or alkyne (**17**) functionalized surfactants. (B/C) Couplings with thiol- (B) or azide-modified (C) payloads allow for modification of copolymers. (D/E) ¹H-NMR analysis indicate quantitative modification.

mixture to form the hydrophobic block (Figure 3A). Previous work has demonstrated that **14** or **15** may be statistically incorporated into the poly(2-methyl-2-oxazoline) chain.^{95,96} The resulting alkene- or alkyne-containing surfactants, **16** and **17** respectively, were characterized by NMR and GPC to contain the desired chemical handles and have M_n and dispersities comparable to surfactant **14**.

With these polymers in hand, we first optimized conditions for thiol-ene and click chemistry. Alkene-containing POx **16** underwent quantitative thiol-ene chemistry by treatment with mercaptoacetic acid (**18**, 5.0 equiv.), Irgacure D-2959 photoinitiator (0.2 equiv.), and irradiation with 365 nm light (output power: 3 x 325mW at 365 nm) overnight to result in modified polymer **19** (Figure 3B,D). Similarly, polymer **17** underwent complete conversion upon treatment with ethylazidoacetate (**20**, 3.0 equiv.), cupric sulfate (0.5 equiv.) and sodium ascorbate (0.3 equiv.), stirring at room temperature overnight to yield modified polymer **21** (Figure 3C,E).

Optimized thiol-ene and click chemistries were then extended from a post-polymerization to a post-emulsification modification strategy. PFC nanoemulsions stabilized by **16** and **17** were prepared following the conditions employed for **8** (Figure 4A). The resulting emulsions were found to be similar in size and polydispersity to **8**, indicating the presence of 15 mol% comonomer did not significantly disrupt the hydrophilic-hydrophobic balance of the surfactant (Figure 4B). Nanoemulsions prepared from **17** that contained alkynes on the surface were fluorescently modified by treatment with azidorhodamine **22** (3.0 equiv.), cupric sulfate (0.5 equiv.) and sodium ascorbate (0.3 equiv.) (Figure 4C). As a control, an emulsion stabilized by the corresponding non-functionalized surfactant (**8**) was exposed to identical conditions. Emulsion sizes were monitored before and after the reaction to confirm that the reagents did not disrupt nanoemulsion stability. The rhodamine absorption of the emulsion solutions exposed to CuAAC conditions was measured before and after dialysis of the samples using UV-Vis spectroscopy. Prior to dialysis, an increased

shoulder on the emulsions containing alkyne suggested aggregation of the fluorophores due to high local concentration on the surface of the droplets (Figure 4D, red solid line). Covalent modification of the surface of the droplets was confirmed after dialysis as the alkyne-containing emulsions retain absorption from the rhodamine while the control emulsions were no longer colored (Figure 4D, dashed line). Emission spectra as well as ¹H-NMR of surfactant isolated post-reaction further confirmed quantitative consumption of the alkyne chemical handles.

Alongside verification that Cu-catalyzed click chemistry was successful at the nanoemulsion surface using a rhodamine dye, we validated that the thiol-ene reaction was a viable approach for post-emulsion modification by modulating the surface charge of the droplets. Changes in surface charge could be quantified by zeta potential analysis, which did not require a purification step. PFC nanoemulsions stabilized by **16** were subjected to photoinitiator (0.8 equiv, Irgacure D-2959) in the presence of thiols (20.0 equiv) methyl mercaptoacetate (**23**),

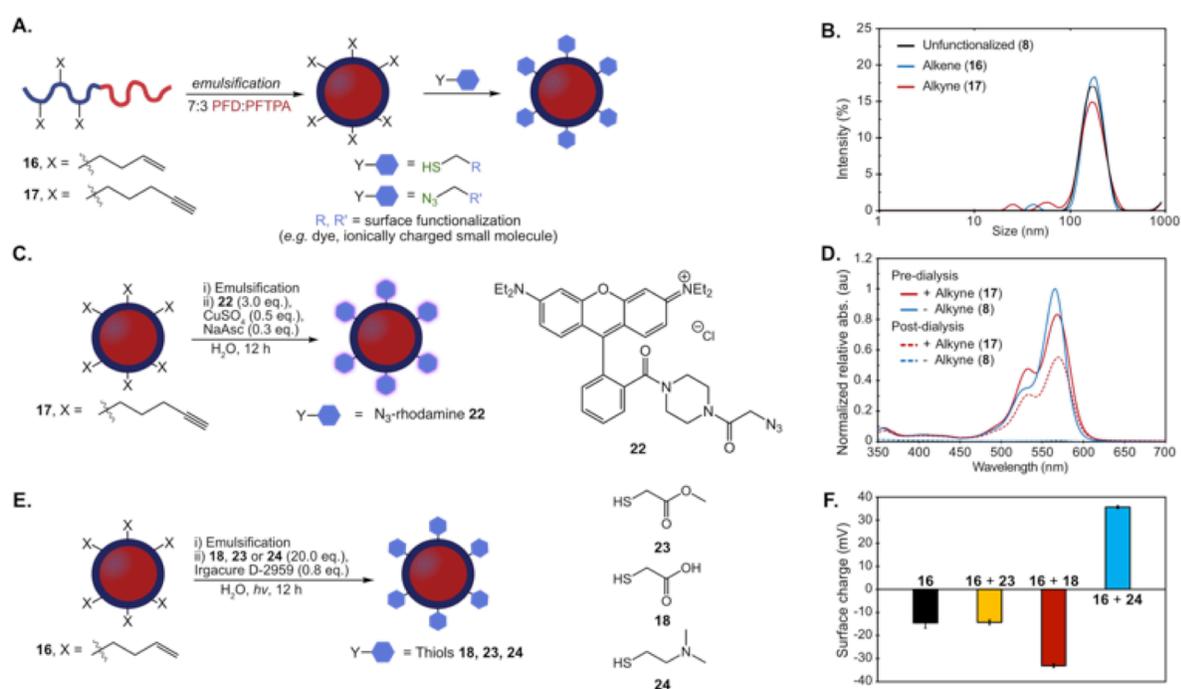


Figure 4. (A) Schematic of post-emulsion modification strategy with surfactants **16** and **17**. (B) Dynamic light scattering data of emulsions formed from **8**, **16**, **17**. Emulsions were prepared with 10 vol% 7:3 PFD/PFTPA and 2.8 wt% surfactant in PBS, diluted 1:100 in MilliQ water and analyzed via DLS. (C) CuAAC chemistry is employed for emulsion surface functionalization with azidorhodamine dye **8**. (D) Emulsions formed from **8** and **17** were subjected to the CuAAC chemistry conditions in (C). After 12 h, the reaction mixtures were diluted 1:100 in MilliQ water and their absorbance was measured (pre-dialysis, solid lines; red = **17**, blue = **8**). The remaining solution was dialyzed in water for 24 h with 3 water changes, at which point the emulsion solution was removed and analyzed by UV/Vis at the approximate concentration as the previous measurements (post-dialysis, dotted lines; red = **17**, blue = **8**). All the data were normalized to emulsion **8** pre-dialysis. (E) Thiol-ene chemistry allows for modulation of zeta potential with ionically charged thiols methyl mercaptoacetate (**23**), mercaptoacetic acid (**18**), and 2-dimethylaminoethanethiol (**24**). (F) Zeta potential of the emulsions before (**16**) and after thiol-ene modification following the conditions in (E). Black = emulsions stabilized by **16**; Yellow = emulsions stabilized by **16** and modified by **23**; Red = emulsions stabilized by **16** and modified by **18**; Blue = emulsions stabilized by **16** and modified by **24**. The surface charge was analyzed by diluting the reaction mixtures 1:100 in MilliQ H₂O and measuring the zeta potential. Data is representative of five replicate measurements. Error bars represent the standard deviation of five measurements.

mercaptoacetic acid (**18**), or 2-dimethylaminoethanethiol (**24**), which will have different protonation states at physiological pH. These solutions were irradiated with 365 nm light overnight and the zeta potential of the samples were measured (Figure 4E/F). As compared to control emulsions stabilized by unmodified **16** (black, Figure 4F), treatment with thiols **23**, **18** and **24** exhibit the expected changes in zeta potential: neutral **23** displays no significant change, acid **18** results in more negatively charged droplets, and amine **24** gives positively charged emulsions. Controls lacking reagents (thiol, light or photoinitiator) corroborate these results, which are further confirmed by NMR analysis of isolated surfactant after the modified emulsions have been disassembled.

Using the thiol-ene post-emulsification strategy, we can modulate the zeta potential of the droplets from +35 to -35 mV at pH 7.4 while keeping the size constant. Notably, it is difficult to obtain emulsions with identical sizes but varied surface charges, as ionic surfactants stabilize interfacial tensions differently compared to non-ionic surfactants.³⁶ We demonstrated that the pre-emulsion functionalization of the surfactants yielded distinct nanoemulsions when compared to post-emulsion modification. Polymers were prepared by reacting **16** with thiols **18**, **23**, or **24**. After isolation, these surfactants were subjected to standard PFC nanoemulsion formation conditions (Figure 5A). We then compared the size and surface charge of emulsions resulting from the pre- or post-emulsification approach to emulsions stabilized by unmodified surfactant **16**. As expected, there was no statistically significant difference in size for emulsions modified with neutral thiol **23** (Figure 5B, yellow). In contrast, differences were observed in the size of anionic and cationic emulsions (Figure 5B, red and blue, respectively). Overall, emulsions formed through a pre-emulsification method varied in size by up to 35 nm, while post-emulsion modification resulted in nanoemulsions with only a 5 nm variance. These results showcase that post-emulsion surface functionalization is a viable approach to decouple the zeta potential of nanoemulsions from their size.

With the ability to access this unique set of nanoemulsions, we performed a systematic study to identify how emulsion surface charge affects cellular uptake. It is known that the size, zeta potential, and surface chemistry of nanoparticles dictate cell uptake *in vitro*,^{74,97} but these experiments have primarily been performed on hard nanomaterials (*e.g.* gold nanoparticles^{74,98,99}), micelles¹⁰⁰⁻¹⁰² or liposomes.^{10,103,104} Results have shown that nanoparticle composition and cell type are also important factors in cell uptake. Thus, extending the scope of uptake studies to include nanoemulsions is necessary.

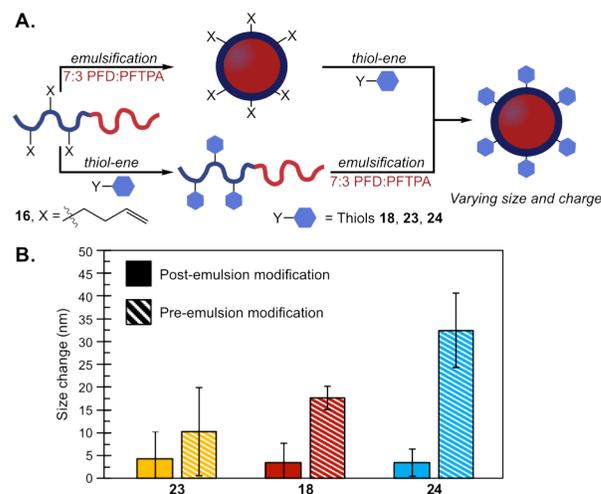


Figure 5. (A) Schematic of emulsions modified through pre- (top) or post- (bottom) emulsion modification methods. (B) Thiol-ene chemistries were performed on surfactant **16** with thiols **18**, **23** or **24** either before (conditions in Figure 3B) or after emulsification (conditions in Figure 4E). The emulsion were diluted 1:100 in MilliQ water and analyzed by DLS. Plotted are the size changes as determined by the absolute difference between size distributions of the resulting emulsions and control emulsions formulated with unmodified **16**. Size data is representative of the average of three independent samples, with three replicate measurements; error bars represent the standard deviation of three independent samples.

We assayed cellular uptake of PFC nanoemulsions in both macrophage and non-macrophage cell lines by loading a fluorosoluble rhodamine dye (**25**, Figure 6B)⁸¹ into the emulsion core. The resulting fluorescent nanoemulsions were incubated with A375 (human melanoma, non-macrophage) or RAW (macrophage) cell lines for 3 hours and, after washing, their degree of fluorescence was quantified by flow cytometry (Figure 6C,D). We performed these studies on emulsions formed from **16** either unmodified or functionalized with **18**, **23**, or **24**, as well as a **1** control.

These experiments showed that cationic nanoemulsions were uptaken in the A375 non-macrophage cell line 250% more than the neutral emulsions and 370% more than the anionic emulsions (Figure 6C). This preference for cationic particles is consistent with other nanomaterial uptake studies.¹⁰⁵⁻¹⁰⁸ Notably, conjugation with neutral thiol **23** resulted in cellular uptake levels similar to that of unmodified **16**, indicating that discrepancies in cellular uptake are due to differences in the physicochemical properties of the nanoemulsions, and not a result of the chemical modification process.

results suggest that the poly(2-methyl-2-oxazoline) surface coverage reduces the non-specific uptake of the emulsions as compared to poly(ethylene oxide). Low levels of non-specific uptake are essential for the active-targeting of nanoparticles.^{112–114} Thus, POx-stabilized emulsions are poised to be versatile materials for targeted delivery.

Finally, we corroborated our quantitative flow cytometry data with microscopy and analyzed the cellular localization of the modified droplets. Previous works have shown that cationic and neutral PFC nanoemulsions undergo endocytosis in both macrophage and non-macrophage cells,^{88,115} while other work has found emulsions to fuse with the cell membrane¹¹⁶. To explore the cellular fate of the POx-stabilized emulsions, we performed colocalization studies with LysoTracker on A375 and RAW cells (Figure 6E,F). The robust colocalization between rhodamine and LysoTracker fluorescence indicate that the nanoemulsions are internalized via endocytosis, and that cells do not use different uptake routes for differentially charged nanoparticles.^{82,105} We also observed an increased interaction with the cell-surface for the cationic particles, presumably due to electrostatic interactions with the anionic membrane.¹⁰⁵

We assayed the cytotoxicity of A375 and RAW cells treated with the different POx emulsions as well as a Pluronic F-68 control. The anionic POx emulsions did not display any statistically significant toxicity in both cell lines over 12 h. Cationic emulsions displayed significant macrophage toxicity and less pronounced, though significant, loss of viability in A375 cells. Interestingly, the unfunctionalized POx stabilized PFC nanoemulsions resulted in substantially larger viability loss in RAW cells than the emulsions that underwent surface modification with neutral thiol **23**. Collectively, our results demonstrate that a post-emulsion functionalization approach is critical for tuning the cellular uptake and viability of these diverse, yet underdeveloped, soft nanomaterials.

CONCLUSIONS:

We demonstrate the use of amphiphilic poly(2-oxazoline)s to stabilize perfluorocarbon-in-water and oil-in-water nanoemulsions. The living nature of the polymerization allows for the controlled addition of functionalizable comonomers into the hydrophilic block of the polymers to facilitate covalent emulsion functionalization. Through incorporation of these functional handles, the ability to attach azide-modified dyes and neutral or charged thiols to the surface of the droplets was achieved. We prepared a set of equal-size yet differentially charged nanoemulsions, which were employed to explore the dependence of cellular uptake on zeta potential in both macrophage and non-macrophage cell lines. We found that cationic emulsions were preferentially uptaken in both cell types. Overall levels of uptake were lower with poly(2-oxazoline) amphiphiles than poloxamers, making the surfactants and

emulsions reported herein promising scaffolds for biomedical applications. Additionally, the ability to modify the surface of nanoemulsions should extend these materials to areas of nanotechnology where control over chemical and physical properties is a prerequisite.

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