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Utilizing Inverse Emulsion Polymerization To Generate Responsive Nanogels for Cytosolic Protein Delivery

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S Supporting Information



ABSTRACT: Therapeutic biologics have various advantages over synthetic drugs in terms of selectivity, their catalytic nature, and, thus, therapeutic efficacy. These properties offer the potential for more effective treatments that may also overcome the undesirable side effects observed due to off-target toxicities of small molecule drugs. Unfortunately, systemic administration of biologics is challenging due to cellular penetration, renal clearance, and enzymatic degradation difficulties. A delivery vehicle that can overcome these challenges and deliver biologics to specific cellular populations has the potential for significant therapeutic impact. In this work, we describe a redox-responsive nanoparticle platform, which can encapsulate hydrophilic proteins and release them only in the presence of a reducing stimulus. We have formulated these nanoparticles using an inverse emulsion polymerization (IEP) methodology, yielding inverse nanoemulsions, or nanogels. We have demonstrated our ability to overcome the liabilities that contribute to activity loss by delivering a highly challenging cargo, functionally active caspase-3, a cysteine protease susceptible to oxidative and self-proteolytic insults, to the cytosol of HeLa cells by encapsulation inside a redoxresponsive nanogel.

KEYWORDS: inverse emulsion polymerization, inverse nanoemulsion, nanogel, redox-responsive nanogels, protein delivery, caspase-3, protease protection

■ INTRODUCTION

With an improved understanding of the human genome, it is clear that genetic imbalances result in various diseases due to abnormalities in transcription, translation, and post-translational modification. To correct these abnormalities, there has been an increased focus in administering biologics to augment a deficient protein or to alter abnormal biochemical pathways.¹ Biologics can be broadly classified into two categories: proteins (including enzymes, regulatory proteins, and antibodies) and nucleic acids (including siRNA, mRNA, miRNA, and DNA). Biologics are preferred therapeutics, due to several advantages over their synthetic small molecule counterparts. Namely, biologics maintain exquisite specificity that often mitigates offtarget toxicities and the potential for efficacy even at catalytic levels. However, systemic administration of biologics as therapeutics does present challenges including possible immunogenicity,² cytotoxicity,³ renal clearance,⁴ premature degradation during circulation,⁵ and poor cellular internalization.6

To circumvent these challenges, various delivery systems have been developed using nanoparticle-based scaffolds including liposomes,^{7,8} polymers,^{9,10} and nanogels.¹¹⁻¹⁶ The biomacromolecular cargo in these systems is typically sequestered by entrapment,¹² covalent conjugation,^{9,17,18} or electrostatic complexation.^{11,19,20} Covalent conjugation of PEG polymers, termed PEGylation,^{21,22} has been widely used to increase the circulation lifetimes of biologics and also protect them from degradative enzymes.^{23,24} However, this strategy is not amenable to all biologics because it requires reactive moieties on the biological molecule, the modification of which may unfavorably alter its activity. Noncovalent methodologies, such as electrostatic interaction, have also been used to complex biologics, commonly with nucleic acids, where positively charged polymers or lipids are used to form poly- and

Received: July 25, 2017 October 16, 2017 Revised: Accepted: October 20, 2017 Published: October 20, 2017 Scheme 1. Cartoon Representation of Caspase-3 Encapsulated within a Redox-Responsive Nanogel and the Resulting Disassembly in a Reducing Environment



lipoplexes.^{25,26} However, the cationic nature of these complexes is associated with cellular toxicity and lytic activity toward red blood cells.²⁷ Nonspecific binding with serum proteins has also been implicated in the rapid clearance of these complexes.²⁸ Considering these limitations, the delivery of biologics by liposomes prepared with noncationic lipids could be a viable approach.²⁹ Nevertheless, a challenge for this approach is that encapsulation of hydrophilic cargo by liposomes is passive and, as such, there is no driving force for the sequestration of hydrophilic cargo into its lumen. Moreover, the use of liposomes as delivery systems is further complicated by their susceptibility to dissociate upon interaction with the plasma membrane of cells.³⁰

Cross-linked nanogels prepared using inverse emulsion polymerization 31,32 (IEP) methodologies have the potential to address many of the limitations outlined above. IEP methodologies generate monodisperse solutions of kinetically stable aqueous (water-in-oil (W/O)) droplets through the use of an aqueous phase, surfactants, and mechanical stirring in an organic medium. Unlike liposomes, hydrophilic cargo can thus be encapsulated into the aqueous interior of inverse nanoemulsions with high cargo loading capacities due to the solvophobic driving forces. The use of covalent cross-links within inverse nanoemulsions, or nanogels, can physically entrap cargo and help prevent leakage when redispersed in an aqueous medium. Through the functional groups that comprise the cross-linker, cargo release from nanogels can be programmed for specific stimulus triggers.^{33,34} Despite these potential advantages, concerns do exist in the formulation of these nanogels through IEP methods. A primary source of apprehension stems from the use of organic solvents and reactive monomers, either of which could cause the biological cargo to irreversibly lose its activity.

In this work, we outline an IEP-based methodology that produces a reliable, redox-responsive protein delivery platform (Scheme 1). Our group has previously exploited the redox sensitivity of disulfide linkages as a stimulus responsive crosslinking moiety in several nanogel technologies, allowing for the release of both protein and small molecule cargos.^{9,17,35} The intracellular reductant concentrations provide a native stimulus for disassembly with maintained particle stability extracellularly. We achieved the formation of a redox-responsive inverse nanoemulsion (nanogel) platform by identifying the key experimental pitfalls in IEP approaches that can lead to loss of biological activity of protein cargos. Systematically, we developed strategies to mitigate these complications. We chose caspase-3 to demonstrate this methodology because of its potential role as a therapeutic, its activity in the reducing cytosol environment, and its sensitivity toward both mild and harsh treatment conditions.^{36,37} The latter feature makes caspase-3 an ideal cargo to test the versatility of our methodology for preserving the activity of the biologic cargo throughout the encapsulation process and carry out its biological function upon delivery. Here, we show that active caspase-3 can be encapsulated within these nanogels and can be subsequently released in response to a redox trigger, for example dithiothreitol (DTT) or a biologically relevant reducing agent, reduced glutathione (GSH). In our optimized process, the released protein is shown to retain its enzymatic activity. We also use a caspase-triggered cell viability assay to demonstrate that the active protein can be delivered inside cells and induce cell death.

EXPERIMENTAL SECTION

Measurements. ¹H NMR spectra were recorded in a 400 MHz Bruker NMR spectrometer with the residual proton of the solvent as internal standard; chemical shifts are reported in parts per million (ppm). Transmission electron microscopy (TEM) was performed on a JEOL 2000FX TEM. Dynamic light scattering (DLS) was performed using a Malvern Zetasizer Nano with a 637 nm laser source with noninvasive back-scattering detected at 173°. Fluorescence measurements were performed using a Molecular Devices SpectraMax M5 fluorescence plate reader.

Materials. L-Cystine, NaCl, NaOH pellets, NaHCO₃, concentrated HCl, hydroxyethyl acrylamide, ammonium persulfate, poly(ethylene glycol) methyl ether acrylate (PEG MEA) M_n 480, tetramethylethylenediamine (TEMED), 2-aminoethyl methacrylate hydrochloride, acryloyl chloride, dithiothriethol (DTT), reduced glutathione (GSH), alamarBlue, and fluorogenic substrate (*N*-acetyl Asp-Glu-Val-Asp-7-amino-4-methylcoumarin, DEVD-AMC) were obtained from commercial sources and used as received, unless mentioned otherwise. Human caspase-3 was expressed in *Escherichia coli* and purified using high performance liquid chromatography (HPLC).

Synthesis of Cystine Bis(acrylamide) (Redox-Responsive) Cross-Linker. The preparation of the redox-responsive cross-linker was adapted from previously established synthetic methods, but with different solvents.^{38,39} 4 g of cystine (16.6

mmol) was taken in a round-bottom flask, and 35 mL of distilled water was added to it. To this was added 2.8 g of NaHCO₃ with continuous stirring. To this cloudy solution were added NaOH pellets (5 g) until the solution became clear, and a pH of \sim 10 was maintained. This solution was then placed under an ice bath, and 4.5 g (50 mmol) of acryloyl chloride added dropwise with continuous stirring while maintaining the pH at 9 using NaOH pellets if necessary. After 3 h, the pH was adjusted to 2.0 by dropwise addition of concentrated HCl (5 mL), while the solution turned white and turbid. The pure product was extracted using ethyl acetate and NaCl, dried over anhydrous Na2SO4, and vacuum-dried to obtain the cystine-bisacrylamide product with an average percentage yield of 57% from two independent trials. It was then analyzed by ¹H NMR (400 MHz, acetone): δ 7.78 (d, J = 7.7 Hz, 2H), 6.41 (dd, J = 17.0, 10.2 Hz, 2H), 6.25 (dd, J = 17.0, 1.6 Hz, 2H), 5.65 (dd, J = 10.1, 1.6 Hz, 2H), 4.99-4.80 (m, 2H), 3.41-3.27 (m, 2H), 3.26–3.07 (m, 2H). NMR spectra: Figure S1. Mass spectra: Figure S2.

Protection of Caspase-3 Protein with Cysteinyl-2pyridyl Disulfide (CPD). 50 equiv of CPD was added to a caspase-3 protein stock solution in PBS pH 7.4 with a protein concentration below 20 μ M and no DTT in solution. This mixture was gently mixed using a pipette and then placed on a rocking plate for 1 h at 4 °C. The unreacted CPD was then removed using centrifuge dialysis using 3000 MWCO Amicon centrifuge dialysis with repeated buffer exchanges. This solution was then further concentrated to a final concentration of 100 μ M.

Synthesis of Inverse Nanoemulsion Nanogels. In a 7 mL glass vial, 0.3 g of Brij L4 was dissolved in 2.5 g of heptane and mixed well using vortex and mechanical stirring to obtain a clear pre-emulsion mixture. In a separate Eppendorf tube, 0.408 mmol of hydroxylethylacrylamide (HEA), 0.005 mmol of cystine diacrylamide, and 10 mg of ammonium persulfate initiator (APS) along with the hydrophilic cargo of interest $(100 \ \mu L \text{ of } 100 \ \mu M \text{ protein, approximately } 0.3 \text{ mg of caspase-3})$ were combined. 100 μ L of PBS pH 7.4 buffer was used to dissolve all these components within the Eppendorf tube through gentle pipette mixing, for a final solution volume of 200 μ L. Nanogels were also previously formulated using different monomers, for example PEG MEA. This nanogel precursor solution was then added to the pre-emulsion mixture to make an inverse nanoemulsion. This solution was then purged with argon for 5 min with gentle stirring to remove any dissolved oxygen. To this was added 25 μ L of tertamethylethylenediamine (TEMED), and the mixture was stirred for an additional 10 min. Encapsulation efficiencies were estimated by observing the remaining free protein present in aqueous solution after nanogel assembly. Consistently, no free or unencapsulated protein was observed in solution after SDS-PAGE analysis.

Extraction of Inverse Nanoemulsion Nanogels. The nanogel solution was centrifuged at 10,000 rpm at 4 °C for 2 min to pellet the nanogel. The heptane solution containing the surfactant was then decanted. The nanogel pellet was then resuspended in 2 mL of heptane and centrifuged again for 30 s, followed by a decanting step. This hexane washing of nanogel pellet was repeated three times to remove the surfactant. Finally, the nanogel pellet was resuspended in 2 mL of PBS pH 7.4 buffer and centrifuged again at 10,000 rpm for 2 min to remove any residual surfactant that is often visualized as an insoluble residue. This nanogel solution was then dialyzed

using 3000 MWCO Amicon centrifuge dialysis at 4 $^{\circ}$ C with multiple PBS changes and then was concentrated to a desired volume. After dialysis, the dry weight of the nanogel solution was calculated to assess the concentration of the nanogel in the solution.

Hemolysis Assay. This assay was executed to assess the hemolysis potential of nanogels. Following a previously reported procedure, in a typical experiment 10^8 red blood cells (RBCs) were incubated with different nanogel concentrations and the final volume of the solution was reconstituted to 1 mL using PBS at pH 7.4.⁴⁰ As a negative control, RBCs were incubated with PBS only. For a positive control 10 wt % of Triton X-100 was used. The RBCs were incubated with nanogels and controls at 37 °C for 1 h. These samples were then centrifuged at 10,000 rpm for 5 min. The supernatant absorbance was recorded at 541 nm using a plate reader, indicating the extent of hemolytic activity. Samples for this assay were analyzed with duplicate technical (same day) replicates; error bars correspond to the percent error calculated from the standard deviation of duplicate wells.

Cell Viability Assessment. Cell viability was tested using HeLa cells and an alamarBlue assay readout. In a typical assay, 10,000 cells per well were preincubated (37 °C at 10% CO₂) for 24 h in a 96-well tissue culture plate using DMEM/F-12 medium with no phenol-red (Gibco, ThermoFisher Scientific), supplemented with 10% FBS, 1% ABAM, and 150 µL of 10 mg/mL gentamicin (Gibco, ThermoFisher Scientific) per 500 mL. After the preincubation, the media in the wells were replaced with 200 μ L of a nanogel-medium solution, with nanogel concentrations ranging from 0 to 2 mg/mL. The cells were then incubated for an additional 24 h. After 24 h, the medium in the wells was then replaced with 100 μL of a 10% alamarBlue-medium solution, which was then incubated for 2 h. Fluorescence measurements were executed by transferring 90 μ L of the sample from a tissue culture plate to a black 96-well flat bottom plate using 560 and 590 nm as the excitation and emission wavelengths. Samples for this assay were also analyzed with duplicate technical (same day) replicates; error bars pertain to the percent error calculated from the standard deviation of both wells.

Caspase-3 Activity Studies. The enzymatic activity of caspase-3 was monitored using the fluorogenic tetrapeptide substrate, DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin), Enzo Lifesciences; Ex 365 nm/Em 495 nm) in an optimal caspase-3 activity buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM CaCl₂, and 10% PEG 400. In an activity assay, the protein sample (nanogel encapsulated caspase-3 or free caspase-3) was first incubated in reductant (100 mM DTT or 10 mM reduced GSH pH 8.5) for 1–3 h and then reacted with the DEVD-AMC substrate at a final concentration of 100 μ M. A basic GSH solution was used to prevent a significant pH change to the caspase-3 activity buffer. The fluorescence measurements were conducted at 37 °C in a 96-well microplate format using a Molecular Devices Spectramax spectrophotometer.

Protein Release Studies. 100 μ L of nanogel sample (containing 2.5 mg of nanogel with 5 μ M estimated protein concentration) was incubated in 100 mM DTT solution overnight. The samples were then concentrated using a 3000 MWCO Amicon centrifuge dialysis tube and analyzed using SDS gel electrophoresis.

Immunoblotting. Purified caspase-3 and nanogel samples were first separated by SDS-PAGE. Proteins were then





Scheme 3. Synthesis of (A) Redox-Responsive Nanogels and (B) Redox-Insensitive (Control) Nanogels



electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were washed for 2 h at 25 °C in a 3% BSA blocking solution containing tris-buffered saline with 0.1% Tween-20 (TBST). The membrane was probed overnight at 4 °C with a mouse anti-human caspase-3 antibody that recognizes the large subunit of caspase-3 (EMD Millipore, MAB 4703). Antibody—antigen complexes were then washed and blotted with goat anti-mouse IgG-peroxidase conjugates at 25 °C (Jackson ImmunoResearch Laboratories). After a final washing in TBST at 25 °C, the membrane was introduced to the enhanced chemiluminescent substrate for horseradish peroxidase (HRP) and detected according to the manufacturer's instructions (ThermoFisher Kit). Images were captured on a ChemiDocMP (Biorad Laboratories Inc.).

RESULTS AND DISCUSSION

Nanogels were synthesized using an inverse emulsion polymer-

ization (IEP) method, using heptane as the continuous phase

and Brij L4 as the surfactant. Scheme 2 shows the steps

involved in the preparation of these nanogels. These inverse nanoemulsions consisted of an aqueous compartment sur-

rounded by a bulk organic phase. Monomers and cross-linkers

were chosen to be hydrophilic such that these molecules

selectively distribute in the dispersed aqueous phase, along with

the hydrophilic protein cargo. In this scenario, a polymerization

reaction in the dispersed phase should cause the protein to be

trapped inside the polymeric nanoparticle. To prepare nanogels

that could release the entrapped protein in an intracellular

3A). Exploiting redox triggers is useful as there is a substantial



Figure 1. (A) Size analysis of polymer and empty redox-responsive nanogels from dynamic light scattering. (B) TEM image of nanogels formulated with a HEA monomer (12x magnification). (C) TEM image of nanogels formulated with a PEG monomer (20x magnification). Scale bars represent 100 nm. (D) Size analysis of redox-responsive nanogel samples after incubating with different concentrations of DTT for 12 h. (E) Percent decrease of redox-responsive nanogel size in response to 10 mM DTT for different time intervals.

difference in the redox potential in the extra- and intracellular environments, due to differences in glutathione concentrations.^{41,42} On the other hand, redox-insensitive nanogels were formulated as a supplemental control through the use of a redox-insensitive bis-acrylamide cross-linker (Scheme 3B). The size, charge, and disassembly characteristics of nanogels prepared with different cross-linkers were monitored (Table TS1).

The cross-linkers are responsible for providing structural integrity to the nanogels. This is supported by the fact that the redox-responsive nanogels, produced by this method, are ~100 nm in the presence of the cystine bis(acrylamide) cross-linker, while the corresponding linear polymer without the cross-linker does not afford a stable nanostructure (Figure 1A). The crosslinks are key to the structural integrity of the nanogels, and since these are made of disulfide bonds, the stability of the nanogel can be compromised by a reducing agent. We tested this possibility by analyzing the change in the hydrodynamic size of cystine bis(acrylamide) based, redox-responsive nanogels using dynamic light scattering (DLS) in the presence of DTT (Figure 1). Indeed, the size of the nanogels reduced from ~100 nm to ~10-20 nm upon incubation with DTT (Figure 1A). Interestingly, the reduced size corresponds to the size of the linear polymer obtained through the IEP process in the absence of the cross-linker. This was further confirmed by transmission electron microscopy (TEM) analysis where the size of the nanogel closely corresponded to the size obtained from DLS (Figures 1B, 1C). The number of particles is greatly reduced upon treatment with a reducing agent (TEM grid with lower number of particles not shown).

To further confirm whether this stimulus responsive behavior is due to the redox sensitivity of the cystine bis(acrylamide) cross-linker, we analyzed the control redox-insensitive nanogels synthesized as mentioned in Scheme 3B. As predicted, the redox-insensitive nanogels do not show any size change upon exposure to a reducing environment (Table TS1). This is one step in confirming that the redox-responsive behavior of the nanogels is associated with the disulfide functionality within the designed cross-linker. Moreover, the kinetics of redox-responsive nanogel disassembly was found to depend on the concentration of the reducing agent (Figure 1D) and duration of incubation (Figures 1E, S3).

The primary goal of this delivery platform is to provide a methodology to entrap biologics inside a nanogel and release them intracellularly, while retaining the biological activity of the encapsulated cargo. To test this possibility, we used the cytosolic protein caspase-3 as the cargo. While using the IEP methodology to encapsulate biologically active proteins, it is important to understand all the reaction components that may affect the protein structure and, thus, protein function and activity. During the nanogel synthesis, the enzyme is exposed to many polymer precursors including the monomer and crosslinker. Since these are often based on substituted acrylamides or acrylates in radical polymerization methods, it is plausible that any reactive thiol present on the protein could undergo a Michael addition reaction with the acrylamide or acrylate based polymer precursors. In fact, caspase-3 is a cysteine protease containing a reactive thiol in its active site. Caspase-3 exists primarily as a dimer, composed of two monomers that each have seven other surface accessible cysteines in addition to the cysteine in the active site, each of which could potentially react with Michael acceptors. The active site cysteine thiol is in fact the most reactive, due to its reduced pK_a , which results from its proximity to the histidine partner of the catalytic dyad. The possibility of a Michael addition reaction between a cysteine residue within caspase-3 and an acrylamide based monomer Scheme 4. (A) Addition Reaction between Caspase-3 and Monomer Leading to Activity Loss. (B) Strategy To Protect Caspase-3 Activity Using a Cysteinyl-2-pyridyl Disulfide (CPD) Protecting Group (PG) Prior to Nanogel Formulation



could covalently modify the active site to irreversibly damage the protein's activity. This was experimentally found to be the case.⁹ To avoid this acrylate induced loss of protein activity, prior to exposing caspase-3 to polymer precursors, we reversibly modified the thiol on the protein by using a cysteinyl-2-pyridyl disulfide (CPD) protecting group for the reactive cysteines.^{9,43} Although this modification can block protein activity, the silencing of activity is temporary as reducing conditions within the cytosol promote deprotection of the catalytic cysteine, allowing caspase-3 to completely regain activity (Scheme 4). It is to be noted that, although caspase-3 has other reactive functional groups (such as 19 amines from lysine residues and 34 carboxylates from glutamate and aspartate residues combined), it is only the reactive thiols of cysteine residues that interact with the polymer precursors to alter the enzymatic activity.

After protecting the active caspase-3 with CPD, we then synthesized caspase-3 encapsulated nanogels and tested their protein releasing behavior in the presence of redox triggers DTT or GSH. As mentioned, intracellular GSH levels are significantly different from extracellular concentrations, as they can reach concentrations up to ~10 mM.^{41,42} Based on SDS-PAGE analysis, we observed that the redox-responsive nanogels successfully entrapped the protein and release the protein cargo only when a reductant (DTT) was present, but no protein was released in the absence of reductant (Figure 2A). Moreover, the redox-responsive nanogels successfully released the caspase-3 cargo when a biologically relevant redox stimulus, GSH, was provided. The resulting protein release from both DTT and GSH was quantified using Western blot analysis. Approximately 5% of the total feed protein was released upon incubation with either reducing agent during the time course of this experiment (Figure 2B).

While it is not possible to accurately determine the total amount of protein encapsulated in the redox-responsive nanogels, this analysis suggests that at least 5% has been stably incorporated. Intriguingly, the GSH-mediated release required a 10-fold lower concentration than DTT, suggesting that these nanogels are eminently compatible with the redox triggers that are present biologically.⁴¹ To further test the versatility of these nanogels, we also encapsulated another protein with different physical properties. Full length PAK2 (FL, p21-Activated Kinase 2) is a monomer of 58 kDa with a pI of 5.7, in contrast

to caspase-3, which is composed of a 17 kDa large subunit and a 12 kDa small subunit with a pI of ~6.0. We observed that PAK2 was likewise efficiently encapsulated in redox-responsive nanogels and subsequently released in response to increased reductant concentrations (Figure 2C). Based on the amount of protein supplied to the inverse emulsion and the dimensions of the nanogels (100 nm diameter), we estimate that each nanogel could contain up to 15 caspase-3 or PAK2 molecules (Calculation CS1) assuming complete incorporation of all supplied proteins during nanogel synthesis. Once we optimized the encapsulation and stimulus-responsive release of proteins within our nanogels, we then assessed the efficacy of these nanogels to retain the cargo's biological activity during the course of the formulation process.

Although treatment of caspase-3 with the CPD protecting group can prevent acrylamide induced protein activity loss, there are also other factors that can affect protein activity in the IEP process. These include the type of organic solvents used, pH of the aqueous fraction used to prepare the water-in-oil (W/O) emulsion, and physical parameters employed such as sonication or mechanical stirring to prepare the nanoemulsions. Care must be taken in varying these factors in such a way that the loss in protein activity is minimized. Organic solvents are known to be associated with protein denaturation, native structure disruption, and thereby loss of activity.⁴⁴ However, using organic solvents is an essential requirement in the IEP method and therefore is a major reason most researchers have avoided IEP methods for encapsulating biologics. While nonpolar solvents such as hexane³⁴ and heptane³² are used to prepare the continuous phase for W/O emulsions, polar organic solvents such as *n*-butanol³⁴ and acetone³² are used in the nanogel extraction process. For protein encapsulation purposes, care must be taken in choosing the most tolerable combination of organic solvents that demonstrate compatibility with the protein of interest. For caspase-3 encapsulated nanogels, we chose to initially follow an existing IEP protocol which employs heptane as a continuous bulk organic phase, Brij-L4 as a surfactant, and *n*-butanol to wash the surfactant after nanogel synthesis.^{12,34} In this procedure *n*-butanol serves to solubilize the Brij-L4 surfactant and break the emulsion, to allow for nanogel extraction. To test the enzymatic activity of the encapsulated caspase-3 after nanogel synthesis, we executed a cleavage assay with a caspase-3 cleavable fluorogenic peptide

Molecular Pharmaceutics



Figure 2. Protein release from redox-responsive nanogels. (A) Coomassie-stained SDS–PAGE demonstrating caspase-3 release. (B) Western blot analysis demonstrating DTT (100 mM) or GSH (10 mM) mediated release. For quantification of the concentration of released caspase-3, a dilution series of human caspase-3 was detected using an anti-caspase-3 antibody that recognizes the large subunit. (C) Coomassie-stained SDS–PAGE demonstrating PAK2 release. For quantification 10 μ L of the indicated concentration of purified PAK2 was included on the same gel.

DEVD-AMC, which mimics a canonical caspase-3 substrate. Although the heptane, Brij-L4, and *n*-butanol method resulted in the synthesis of protein-encapsulated nanogels with good yields, the activity of caspase-3 was completely lost during the process. We found that *n*-butanol, used in the nanogel extraction process, had a detrimental effect on the caspase-3 activity (Figure 3A). Interestingly, although hydrophobic, apolar solvents are likely to play a role in these processes,⁴⁴ heptane did not show any significant effect on caspase-3 activity.

To avoid the use of *n*-butanol, we employed the use of centrifugation to extract the nanogels. Since the nanogels are



Figure 3. Optimized pH and solvent conditions for encapsulation and release of active caspase-3. (A) The activity of caspase-3 is impacted by exposure to different solvent conditions. Heptane:butanol represents an organic solvent mixture. After treatment with various solutions, caspase-3 activity assays were conducted in the optimized caspase-3 activity buffer. (B) Activity of caspase-3, assayed at pH 7.5 in the optimized caspase-3 activity buffer, after preincubation at different pH conditions. (C) Caspase-3 activity in samples of 50 nM free CPD-treated caspase-3 or 50 nM caspase-3 recovered from nanogels in the presence of reductant. For panel C, the concentration of caspase-3 released from redox-responsive nanogels was determined from Figure 2B and the activity was assayed in triplicate, with error analysis corresponding to the standard error of the mean. In all panels, a caspase-3 DEVD-AMC fluorogenic substrate was used.

associated with trace amounts of water, these particles can be pelleted with high-speed centrifugation of the nanoemulsion solution (which contains nanogel, surfactant, and heptane). After this step, heptane was decanted and the nanogels were pelleted and washed with heptane several times to remove any residual surfactant. With this protocol, we recovered only a small amount of activity ($\sim 1-2\%$).

Upon further analysis of our formulation protocol, the other factor that we identified as a potential contributor to the activity loss was pH. In the process of preparing the W/O emulsion, the aqueous fraction consists of monomer, cross-linker, ammonium persulfate (APS) initiator, and the protein of interest in a pH 7.4 PBS buffer. Although buffer was used to maintain an optimum pH in the aqueous fraction, we observed that the APS initiator significantly lowered the pH of the solution to ~3.0. We recognized that this change in pH could cause a loss in caspase-3 activity. We found that, indeed, caspase-3 lost its activity even after just a brief exposure to acidic pH (Figure 3B), which is likely due to a pH induced protein structural change. 45,46

To overcome the problem of acidification due to the APS initiator, we used a 1.0 M NaHCO₃ solution to dissolve the



Figure 4. (A) Cell viability assay upon incubation of caspase-3 encapsulated nanogels (redox-responsive and redox-insensitive control nanogels) and free, unencapsulated caspase-3. (B) Cellular viability of HeLa cells after incubation with nanogels at different concentrations. (C) Hemolysis assay of red blood cells in the presence of nanogels at different concentrations. All error bars correspond to the percent error calculated from the standard deviation of duplicate wells.

nanogel precursors instead of PBS pH 7.4, to maintain a slightly basic pH. Using this method for nanogel preparation, we are able to recover active caspase from the nanogel. Recall that we observed that \sim 5% of the feedstock caspase-3 was released from the nanogel under our experimental conditions (Figure 2B). We compared the activity of released caspase-3 from redox-responsive nanogels, via DTT or GSH, to the activity of free caspase-3 treated with the CPD protecting group (Figure 3C). We observed similar levels of caspase-3 activity in both reductant-treated samples, but we in fact recovered slightly greater activity (1.5- to 2-fold) of the caspase-3 released from the nanogels compared to the free control. This apparent increase in activity could be the result of errors in the estimation of the concentration, or due to the fact that encapsulation of caspase-3 within the nanogels may prevent self-proteolysis. Alternatively, PEG and other crowding agents are known to increase caspase activity.^{46,47} It is possible that the disassembled nanogels may be acting as crowding agents, similar to that observed for free caspase-3 activity in the presence of additional PEG (Figure S4). Overall, these compelling data show that caspase-3 can be reversibly encapsulated, protected during encapsulation, and released in an active manner by a biologically relevant reductant.

During this work, we prepared the control redox-insensitive nanogels, which were composed of a bis(acrylamide) crosslinker. These redox-insensitive nanogels do not release the protein upon exposure to reducing agent (Figure S5), but we do observe some activity from the encapsulated caspase-3 (Figure S6). This activity suggests that the peptide-based fluorogenic substrate is able to diffuse into the nanogel and access caspase-3 for cleavage. This feature, in the redoxinsensitive nanogels, suggests that there may be subtle differences in the structures of the redox-insensitive and redox-responsive nanogels, as the redox-sensitive nanogels show essentially no activity unless reductant is present. On the other hand, because some activity can be observed of the redoxinsensitive control nanogels, the permittivity of these nanogels may offer new opportunities for future caged catalytic reaction applications.

We also sought to demonstrate disassembly of caspase-3 encapsulated redox-responsive nanogels in the presence of biologically relevant reductant GSH (Figure S7). We have

observed that nanogel disassembly by GSH, as observed by DLS, was not as effective as DTT. However, when we added the surfactant Triton X-100 (to a concentration of 0.3%) in addition to GSH, we observed disassembly of the 100 nm nanogels to the same 10-20 nm sized particles as we observed with DTT (Figures 1A, 1D, S3). There was no induced disassembly by Triton X-100 alone; we predict that the micelleforming surfactant is simply localizing the concentration of GSH to the nanogels. Given the wide range of intracellular metabolites and lipids present intracellularly, we anticipate that intracellular GSH will be sufficient to release proteins from the redox-sensitive nanogels. For intracellular delivery, protein release is critical as the typical substrates for these proteases are much larger than the tetrapeptide used in this assay. Thus, GSH induced release was confirmed by the results of our cell toxicity assays (vide infra for cellular activity).

Caspase-3 is an apoptotic protein that causes cell death upon its delivery into the cytosol. If cell internalization combined with cytosolic reductant concentrations causes disassembly of our redox-responsive nanogels and, thus, release of the active (deprotected) encapsulated caspase-3 in the cytosol, then apoptosis will be initiated, propagating events within the programmed cell death pathway. Indeed, we found that, at a concentration of ~0.25 μ M caspase-3 (encapsulated in the redox-responsive nanogel), <20% of the cells survived (Figure 4A, redox-responsive nanogel lane). If our delivery hypotheses are correct, then it is the redox-responsive character of the nanogel that is causing the executioner caspase to be released in its active form within the cytosol. It is critical that we evaluate this hypothesis and confirm that the cell death observed is as we hypothesize. For this, we tested the effect of the redoxinsensitive control nanogels that also had caspase-3 encapsulated; no discernible cell kill was observed from these (Figure 4A, control nanogel lane). This control experiment confirms our earlier assertion that the activity, observed with a small molecule fluorogenic substrate (Figure S6), does not translate to intracellular activity where the substrates for these apoptotic proteins are much larger and would require release of the caspase cargo from the nanogel. The cell viability assays were carried out in technical duplicate, by incubating the nanogels (with a concentration of 1.25 mg/mL) with cells at 37 $^{\circ}$ C for 6

Molecular Pharmaceutics

h, after which the cellular viability was quantified using an alamarBlue assay.

Of course, two more controls are required for appropriate interpretation of these results: free caspase internalization and apoptosis induction as well as inherent toxicity of the redoxresponsive nanogels themselves. Caspase-3 is a neutral protein, with no known cell-penetrating capabilities. Moreover, in our previous studies we observed that free, unencapsulated caspase-3 does not have the ability to enter cells and promote cell death, even at high protein concentrations.⁹ Therefore, as expected, at a comparable concentration of free caspase-3 to that of the caspase-3 cargo inside the nanogel, there is no discernible cell death (Figure 4A, caspase-3 lane). Next, we tested the toxicity of the nanogels prepared without caspase-3. The nanogels were found to be nontoxic, even at concentrations far exceeding previous experiments (Figure 4B). Also, we tested the in vivo applicability of these nanogels by investigating their hemolytic potential. Upon incubating these nanogels with human red blood cells (RBC), we did not observe any nanogel induced hemolysis (Figure 4C), suggesting that exposure to the nanogels does not compromise the cell membrane. Taken together, these results demonstrate that the redox-responsive nanogels are capable of delivering proteins into the cytosol and that the redox-responsive liberation of the protein is critical for its activity inside the cells.

CONCLUSION

Solvophobic driving forces provide a potentially direct and effective approach for the encapsulation of hydrophilic molecules, such as proteins, because both the carrier and the cargo are hydrophilic. For this reason, inverse emulsion polymerization (IEP) is an obvious strategy to generate nanoscale protein delivery vehicles. However, this approach has been largely avoided in the literature due to the notion that exposure of structurally fragile proteins to organic solvents will compromise their function. In this work, we show that these complications can be overcome by systematically analyzing and circumventing the factors that can damage protein activity. We show here the following: (i) When confronted with complementary reactivity profiles between functional groups in the protein and polymerizable monomers, one of the functional groups can be protected through a design that allows for unmasking (deprotection) under the same conditions for nanogel disassembly. (ii) Although conventional wisdom would suggest that the apolar continuous phase would cause proteins to unfold and lose activity, it is the polar organic solvent, which is used to separate the nanogels, that causes this problem. We circumvented this by using a physical method (centrifugation), rather than chemical, for nanogel extraction. (iii) The ammonium persulfate initiator can depress the pH of the dispersed phase and cause a protein to lose activity. We addressed this issue by simply ensuring that the pH is maintained by adding a mild base. After overcoming these confounding issues, it is clear that inverse emulsion polymerization methods can indeed be a promising approach for encapsulating and delivering proteins, with maintained biological activity. Considering the versatility of this approach, and its biocompatibility, this delivery platform has further potential to deliver nucleic acids and simultaneous delivery of both proteins and nucleic acids into the cytosol, which is part of the ongoing research in our laboratory. More generally, we believe that the insights presented here provide a template for

optimizing delivery vehicles for a large number of globular proteins.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharma-ceut.7b00643.

¹H NMR and mass spectra and response of redoxresponsive nanogels (PDF)

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Notes

The authors declare no competing financial interest.

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