Biocompatible Single-Chain Polymer Nanoparticles for Drug Delivery—A Dual Approach

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Supporting Information

ABSTRACT: Single-chain polymer nanoparticles (SCNPs) are protein-inspired materials based on intramolecularly cross-linked polymer chains. We report here the development of SCNPs as uniquely sized nanocarriers that are capable of drug encapsulation independent of the polarity of the employed medium. Synthetic routes are presented for SCNP preparation in both organic and aqueous environments. Importantly, the SCNPs in organic media were successfully rendered water soluble, resulting in two complementary pathways toward water-soluble SCNPs with comparable resultant physicochemical characteristics. The solvatochromic dye Nile red was successfully encapsulated inside the SCNPs following both pathways, enabling probing of the SCNP interior. Moreover, the antibiotic rifampicin was encapsulated in organic medium, the loaded nanocarriers were rendered water soluble, and a controlled release of rifampicin was evidenced. The absence of discernible cytotoxic effects and promising cellular uptake behavior bode well for the application of SCNPs in controlled therapeutics delivery.

KEYWORDS: single-chain polymer nanoparticles, controlled drug delivery, thiol-Michael addition, thiol polymers, drug encapsulation

INTRODUCTION

Single-chain polymer nanoparticles (SCNPs) are prepared through exclusive intramolecular cross-linking of polymer chains and have been developed as promising uniquely sized nanocarrier systems over the past 2 decades. The intramolecular chain collapse gives access to extremely small polymeric nanoparticles (~10 nm), unparalleled by other means of preparation. In view of anticipated applications as drug carriers and protein-mimicking systems, a number of strategies to prepare the water-soluble SCNPs has been brought into focus in the recent past, including the postformation functionalization of carboxylic acid polymers with benzyl or tert-butylisocarbonyl protecting groups, or alternatively the use of amphiphilic copolymers such as poly(N-isopropylacrylamide), poly(2-(N,N-dimethylamino)ethyl methacrylate), and polyethylene glycol. Several strategies have been developed to covalently cross-link the polymers intramolecularly directly in water, for example, via amidation of glutamic acid, via thiol-Michael addition, or via tetrazole–ene cycloaddition.

To investigate the potential of SCNPs as drug carriers, dye molecules, as well as therapeutic cargos such as doxorubicin, have been successfully encapsulated into the SCNPs. To our knowledge, a nanoparticle system for therapeutics encapsulation independent of their lipophilicity with appreciable encapsulation efficiencies is still pending.

We recently demonstrated the rapid and efficient synthesis of SCNPs via a phosphine-induced thiol-Michael cross-linking in an organic medium. The thiol-Michael reaction can be readily performed in the organic medium without the need for a high temperature or metal catalysts but also takes place in a basic aqueous medium and therefore poses little restriction with respect to the reaction medium.

Herein, we report on the dual-pathway synthesis in both the aqueous and organic phases of water-soluble SCNPs, enabling a polarity-independent therapeutics encapsulation and a subsequent release under physiologically relevant conditions (Scheme 1). The cytotoxic effects of the SCNPs are evaluated on human cervical cancer (HeLa) and human brain endothelial (hCMEC/D3) cells. hCMEC/D3 cells are further explored as a model for...
“Starting from the polymer P₀ (soluble in organic solvents), nanoparticles are prepared in dichloromethane (NP₀w, organic pathway), or the polymer is hydrolyzed to its water-soluble analogue (PW) to serve as a precursor for nanoparticle formation in an aqueous solvent (NP₀w, aqueous pathway). Nanoparticles NP₀w are also hydrolyzed to render them water-soluble (NP₀wh).

**EXPERIMENTAL SECTION**

**General Procedures for Thiol Aminolysis of the Copolymers.** The copolymer (500 mg) (i.e., 0.30 mmol equiv thiol monomer) was dissolved in 10 mL of the solvent [tetrahydrofuran (THF) for the organic route and dimethyl sulfoxide (DMSO) for the aqueous route] and purged with nitrogen for 10 min in a sealed round-bottom flask. Under nitrogen flow, hydrazine monohydrate (29 μL, 0.60 mmol, 2.00 equiv) was added. The solution was stirred for 60 min in the case of P₀ and 30 min for PW. The copolymer solutions were filtered and immediately used in nanoparticle formation.

**Nanoparticle Formation in an Organic Solvent (NPw).**

The copolymer (500 mg) (i.e., 0.30 mmol equiv thiol monomer) was dissolved in 10 mL of the solvent [tetrahydrofuran (THF) for the organic route and dimethyl sulfoxide (DMSO) for the aqueous route] and purged with nitrogen for 10 min in a sealed round-bottom flask. Under nitrogen flow, hydrazine monohydrate (29 μL, 0.60 mmol, 2.00 equiv) was added. The solution was stirred for 60 min in the case of P₀ and 30 min for PW. The copolymer solutions were filtered and immediately used in nanoparticle formation.

**P₀**. 1H NMR (400 MHz, CDCl₃) δ ppm: 4.40–4.22 (br, CH), 4.14–3.65 (br, m, CH₂), 2.80–2.67 (br, CH₂), 2.12–1.71 (br, 1.69–1.45 (br), 1.48–1.32 (d, (CH₃)₂), 1.14–0.78 (br).

**NPw**. 1H NMR (400 MHz, DMSO-d₆) δ ppm: 4.92 (s, OH), 4.68 (s, OH), 4.33–4.22 (br, CH₂), 4.05–3.16 (br, m, CH, CH₂), 2.11–1.56 (br), 1.17–0.54 (br).

**Nanoparticle Formation in an Organic Solvent (NP₀w).**

The copolymer (500 mg) (i.e., 0.25 mmol equiv thiol monomer) in THF (10 mL) was dropwise added to the continuously stirred cross-linker solution over 30 min. After 4 h of stirring, 2.2 mL of dimethylaminoethyl acrylate (DMAEA) (16 mmol) was added, and the solution was left stirring overnight. Subsequently, the solution was filtered and concentrated under a reduced pressure. The obtained solution was dialyzed against demi-water, filtered, and freeze-dried to obtain a white lyophilizate (~300 mg).

**NP₀w**. 1H NMR (400 MHz, DMSO-d₆) δ ppm: 6.41–4.29 (CH), 6.27–6.12 (CH), 6.03–5.85 (CH), 4.92 (s, OH), 4.68 (s, OH), 4.31–3.03 (br, m, CH, CH₂), 2.88–2.59 (br, m, CH₂), 2.11–1.56 (br), 1.17–0.54 (br).

**Nile Red Encapsulation (NPw-NR and NP₀w-NR).** For the encapsulation of Nile red (NR) in SCNPs, NR was added during the above-described SCNP formation procedure. For the formation in an organic solvent, NR was added to the dichloromethane phase (0.4 mg/mL) and the obtained solution was dialyzed against water followed by filtration and freeze-drying to obtain a dark purple lyophilizate. For NP₀w-NR, the nanoparticles were hydrolyzed through the addition of 10 mL of aqueous HCl solution (8 M). Both nanoparticle systems, NPw-NR and NP₀w-NR, were dialyzed against water followed by filtration and freeze-drying to obtain a dark purple lyophilizate.
Complete hydrolysis was conducted with the cross-linker. As 


1H NMR spectroscopy does not allow distinguishing between intra- and intermolecular reactions, GPC (Figures S1 and S2). As the thiol-Michael addition in an aqueous environment proceeds most efficiently under basic conditions,16 SCNP formation was performed with TCEP as an activator in CBB (0.1 M, pH 9) with oligoethylene glycol diacrylate (Mw = 258 Da) as a water-soluble cross-linker and N,N-DMAEA as an endcapper. Successful thiol-Michael addition was confirmed by 1H NMR spectroscopy (Figure S1). The GPC measurements on both polymers PW and nanoparticles NPW revealed relative size reductions of 10–30% depending on the polymer length. Longer polymers result in larger size reductions (Table S1 and Figure 1a,b), presumably because of the higher number of cross-links. The dependency of nanoparticle size on the precursor polymer is an indication of SCNP formation and is in line with our previous findings for thiol-Michael addition-based SCNPs.14

Dynamic light scattering (DLS) measurements revealed comparable sizes for all NPW nanoparticles, around 4.0 nm in hydrodynamic radius (rH), whereas the sizes of the polymer precursors depend on the molecular weight, ranging from 3.6 to 7.2 nm, hence demonstrating a higher relative size reduction for longer polymers (Table S1 and S2). Diffusion-ordered spectroscopy (DOSY) NMR spectroscopy experiments further support the DLS measurements, evidencing a reduction in rH from 8.8 to 7.9 nm in DMSO (Figure S4). Small-angle X-ray scattering (SAXS) experiments confirm a size reduction by the cross-linker-induced chain collapse and a radius of gyration of 3.9 nm for NPW (Figure S5 and Table S3). Fitted SAXS measurements further reveal a decrease in the scaling exponent ν in DMSO when comparing the polymer (0.58) with the SCNPs (0.44). As described by the Flory exponent, the polymer corresponds to a self-avoiding chain (ν = 0.6), while the SCNPs approach the behavior of coiled, spherical structures (ν = 1/3).18 Further analysis with a triple-detector GPC system [multilangle light scattering (MALS), refractive index, and viscometer] in DMF revealed a decrease in the hydrodynamic radius and intrinsic viscosity upon cross-linking while molecular weight remained unaltered (Table S4).

Scanning transmission electron microscopy (STEM) imaging of negatively stained NPW shows uniform round objects ca. 6.5 nm in radius (Figure 1c). This size is larger than the sizes determined by DLS and SAXS, but in agreement with the particles in a dried state.14,20

Hydrolysis of the solketal moieties of the nanoparticle NP_O was performed to obtain the water-soluble SCNPs NP_O via the organic pathway. Because hydrolysis requires relatively strong acidic conditions, the integrity of the intramolecular cross-links formed during the thiol-Michael addition needs to be verified. Upon comparing the hydrolyzed nanoparticles NP_O with the nanoparticles NP_W based on the water-soluble precursor polymer PW, both 1H NMR and FT-IR measurements show comparable spectra, and no signs indicating the formation of carboxylic acids due to the hydrolysis of ester bonds were observed (Figures S1 and S9). The molecular weights of the hydrolyzed polymer PW and the nanoparticle NP_O are in the range of 40 kDa.
same range as the molecular weights observed for the corresponding polymer P₀ and the nanoparticle NP₀ before hydrolysis, with an apparent size reduction of 11% (Figure S10 and Table S5).

In order to probe the SCNP formation process, the solvatochromic dye NR was encapsulated by carrying out SCNP formation in the presence of NR, via both the organic and the aqueous pathways. NR is poorly soluble and barely fluorescent in water; however, upon encapsulation, the fluorescence intensity of the lyophilized NR-containing SCNPs (NP⁰-NR) in water increased markedly, while the emission blue-shifted in comparison to free NR, indicating a more hydrophobic environment of NR. Successful encapsulation of NR in both NPₘ and NP⁰ was evidenced by the coelution of nanoparticles and NR in GPC measurements (Figures 2a and S10b). Further, the fluorescence spectra of the encapsulated NR are red-shifted, demonstrating that NR is located in a more hydrophilic environment than DMF. The red shift is more pronounced for NPs prepared via the organic pathways. NR is poorly soluble and barely fluorescent in water; however, upon encapsulation, the fluorescence intensity of the lyophilized NR-containing SCNPs (NP⁰-NR) in water increased markedly, while the emission blue-shifted in comparison to free NR, indicating a more hydrophobic environment of NR. Successful encapsulation of NR in both NPₘ and NP⁰ was evidenced by the coelution of nanoparticles and NR in GPC measurements (Figures 2a and S10b). Further, the fluorescence spectra of the encapsulated NR are red-shifted, demonstrating that NR is located in a more hydrophilic environment than DMF. The red shift is more pronounced for NPs prepared via the organic pathway (NP⁰) most likely because of a higher NR concentration during the formation. It should be noted that NR has only limited water solubility, which is why no release study was performed for NR.

In order to evaluate SCNPs as drug carriers, Rif, a broad-spectrum antibiotic with an excellent bactericidal activity, particularly used in the fight against tuberculosis and pneumococcal meningitis, was encapsulated in NP⁰ nanoparticles. GPC analysis revealed the coelution of Rif and NP⁰ nanoparticles, as observed by a UV–vis absorbance band characteristic of the encapsulated Rif (Figure S13). UV–vis spectroscopy indicates that the NP⁰-Rif nanoparticles contain approximately 16 wt % Rif, which corresponds to an entrapment efficiency of 81%. It should be noted that not all free Rif can be removed without also removing the cargo Rif. Subsequently, drug release was assessed through dialysis of the obtained NP⁰-Rif nanoparticles and tracking Rif release via UV–vis measurements. After a burst release of Rif in the first 2 h (40% release), a sustained release of Rif from NP⁰-Rif was observed, as compared to a Rif control solution (Figure 2b). Although the majority of Rif (80%) of the control solution was dialyzed out at the first time point (2 h), the NP⁰-Rif solution still contained over 60% of the original Rif content. Electrospray ionization mass spectrometry confirmed that Rif remained unaltered after encapsulation, acid hydrolysis, and release (Figure S14).

An important prerequisite for use as a drug carrier is biocompatibility. Both polymer P₂ and nanoparticle NPₘ were evaluated for cytotoxicity with a cell viability assay on HeLa (Figure S6) and on hCMEC/D3 cells (Figures 3 and S7).

Both cell lines maintained their metabolic activities after incubation with Pₘ and NPₘ for 48 h. Figure 3. Metabolic activity of hCMEC/D3 cells after incubation with Pₘ and NPₘ for 48 h.

A common issue with the cell uptake of nanoparticles is degradation by lysosomes. Importantly, lysosome containing of hCMEC/D3 cells did not reveal the colocalization of nanoparticles with lysosomes (Figure S18b), although the CLSM images suggest vesiculation of the nanoparticles and hence uptake via endocytosis. The uptake mechanism and final destination of the SCNPs are currently under investigation. Because the nanoparticles are able to bypass the lysosomes, their
ability as a drug delivery agent was further investigated by employing fluorescent NR as a model compound. DTAF-labeled NP_{OH}-NR was chosen because of the higher drug loading obtained via the organic pathway. As observed in Figure 4, NR and DTAF are colocalized in the cells. It should be noted that the sample contains a non-negligible amount of non-encapsulated NR. Nevertheless, the colocalization of cargo and nanoparticles hint toward SCNPs as a successful drug delivery system. As the aforementioned release studies with NR proved unsuccessful, the release inside the cytosol may be hampered as well.

CONCLUSIONS

Making use of thiol-Michael cross-linking and solketal as an adaptable moiety, both organic and aqueous pathways toward water-soluble SCNPs have been presented. A combination of characterization methods demonstrated the formation of well-defined particles approximately 3–5 nm in radius. Cell viability studies demonstrated no cytotoxicity effect even at elevated concentrations. Furthermore, uptake by hCMEC/D3 cells was demonstrated with DTAF-labeled SCNPs. In combination with drug encapsulation and release, this SCNP system offers the dual-preparation pathway—the opportunity to encapsulate drug molecules irrespective of their lipophilicity. These results highlight the potential of SCNPs as a biomaterial and in particular as a drug delivery system to brain tissues. Current efforts are focused on the in vivo evaluation of these promising drug carriers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b07450.

Materials and methods, Ellman’s assay, cell toxicity/cellular uptake experiments, ¹H NMR, FT-IR, DOSY-NMR, SAXS, GPC, electrospray ionization mass spectrometry, fluorescence, UV–vis absorption, and FACS measurements (PDF)

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