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

A. Pia P. Kröger, Naomi M. Hamelmann, Alberto Juan, Saskia Lindhoud, and Jos M. J. Paulusse

INTRODUCTION

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

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INTRODUCTION

Single-chain polymer nanoparticles (SCNPs) are prepared through exclusive intramolecular cross-linking of polymer chains and have been developed as promising uniquely sized nanoparticle 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-butylloxycarbonyl 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 or via thiol-Michael addition, or via tetrazole—ene cycloaddition. 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...
The solution of deprotectedequiv) were added to 100 mL of nitrogen-purged dichloromethane.

3.65 (br, m, CH₂), 2.80 equiv) was added. The solution was stirred for 60 min in the case of pathway). Nanoparticles are also hydrolyzed to render them water soluble (NP_{OH}).

Drug transport to the central nervous system in cellular uptake studies with SCNP.

**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 NP and 30 min for PW. The copolymer solutions were filtered and immediately used in nanoparticle formation.

**NP** 1H NMR (400 MHz, CDCl₃) δ: 4.20–4.22 (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).

**PW** 1H NMR (400 MHz, DMSO-d₆) δ: 3.16 (br, m, CH, CH₂), 2.11 (br), 1.71 (br), 1.69 (br), 1.45 (br), 1.17–0.54 (br).

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

**NPOH** 1H NMR (400 MHz, DMSO-d₆) δ: 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), 2.21–1.56 (br), 1.17–0.54 (br).

**Nile Red Encapsulation (NPW-NR and NPOH-NR).** For the encapsulation of Nile red (NR) in SCNP, NR was added during the above-described SCNP formation procedure. For the formation in an organic solvent, NR was added to the dichloromethane phase (0.04 mg/mL) and during the formation in the aqueous solvent, NR was added to the DMSO phase (1 mg/mL) as NR is barely soluble in water. Subsequently to the reaction, the reaction mixtures were concentrated. For NP_{O-H}-NR, the nanoparticles were hydrolyzed through the addition of 10 mL of aqueous HCl solution (8 M). Both nanoparticle systems, NPW-NR and NPOH-NR, were dialyzed against water followed by filtration and freeze-drying to obtain a dark purple lyophilizate.

For excitation spectra, the emission was detected at 615 nm. For NPW-NR and NPOH-NR, the samples were dissolved in dimethylanisole (DMF) and analyzed by gel permeation chromatography (GPC) coupled with a fluorescence detector. For NPW-NR, the eluents were excited at 550 nm, and for excitation spectra, the emission was detected at 615 nm.
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) and by Fourier transform infrared (FT-IR) spectroscopy (Figures S3 and S4) was employed to provide thiol moieties for Michael cross-linking, were prepared via reversible addition-fragmentation chain transfer polymerization. The incorporation ratios of the obtained copolymers (Pv) with molecular weights between 30 and 70 kg/mol and low polydispersities (PDI ≈ 1.10) matched well with the feed ratios of 10% of XMA.

Prior to SCNP formation in the organic solvent, xanthate moieties were deprotected via thiol aminolysis with hydrazine. 1H NMR spectroscopy confirmed full deprotection within 30 min through the disappearance of the ethyl xanthate signals. Pv was cross-linked via thiol-Michael addition under continuous addition of the deprotected polymer to a cross-linker solution containing a phosphine initiator as described previously. The residual thiol moieties were passivated by excess methyl acrylate, and product nanoparticles (Pv) were isolated via precipitation. Separate signals corresponding to the methyl group of methyl acrylate at 3.71 ppm and to the butyl ester moiety of butanediol diacrylate at 1.75 ppm are identical in the 1H NMR spectrum (Figure S1). Although both signals overlap to some extent with other signals, approximately half of the thiol react with the cross-linker. As 1H NMR spectroscopy does not allow distinguishing between intra- and intermolecular reactions, GPC was employed (Table S5 and Figure S10). The comparison of the elution times of polymer Pv and nanoparticle Pv revealed a relative size reduction of 23%, indicating a chain collapse through intramolecular cross-linking.

To achieve water-soluble nanoparticles directly from the polymers, the above-described SCNP formation process was also carried out in an aqueous medium. To render polymer Pv water soluble, the xanthate moieties on the polymer were hydrolyzed to the corresponding diglycol groups under acidic conditions to achieve a water-soluble copolymer (Pw).

Complete hydrolysis was confirmed by 1H NMR spectroscopy and by Fourier transform infrared (FT-IR) spectroscopy (Figures S1 and S2). As the thiol-Michael addition in an aqueous environment proceeds most efficiently under basic conditions, SCNP formation was performed with TCEP as an initiator in CBB (0.1 M, pH 9) with oligo ethylene 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 Pw released 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.

Dynamic light scattering (DLS) measurements revealed comparable sizes for all 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 Pw (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). Further analysis with a triple-detector GPC system [multiance 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 nanoparticles 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.

Hydrolysis of the solketal moieties of the nanoparticle PO was performed to obtain the water-soluble SCNP’s PNH 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 PNH with the nanoparticles Pw 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 PNH are in the range of 30–80 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 (NP0H-NR) in water increased markedly, while the emission blue-shifted in comparison to free NR, indicating a more hydrophilic environment of NR.21,22 Successful encapsulation of NR in both NPw and NP0H 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 (NP0H) 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 during the formation. It should be noted that NR 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 NPw 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 Pw and NPw for 48 h.

Figure 3. Metabolic activity of hCMEC/D3 cells after incubation with Pw and NPw for 48 h.

A common issue with the cell uptake of nanoparticles is degradation by lysosomes.28 Importantly, lysosome containing 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

Drug release was assessed through dialysis of the obtained NP0H-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 NP0H-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 NP0H-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).
ability as a drug delivery agent was further investigated by employing fluorescent NR as a model compound. DTAF-labeled NPOH-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 with 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)

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: j.m.j.paulusse@utwente.nl.

ORCID

A. Pia P. Kröger: 0000-0001-7031-0598
Naomi M. Hamelmann: 0000-0002-7126-4818

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Figure 4. CLSM images of hCMEC/D3 cells (a) after incubation with DTAF-labeled NPOH (green) for 20 h stained with Alexa Fluor 647 phalloidin (red) and 4′,6-diamidino-2-phenylindole (DAPI) (blue); (b) after incubation with DTAF-labeled, NR-loaded NPOH for 20 h stained with DAPI (blue).

J.M.J.P. and A.P.P.K. gratefully acknowledge the funding from the Netherlands Organization for Health Research and Development (ZonMw, project number 733050304). S.L. and J.M.J.P. thank the Diamond Light Source for the beam time (SM16050-1) and Dr. Katsuki Inoue and Dr. Robert Rambo for their involvement in the SAXS experiments. Mark Smithers is thanked for the STEM measurements and Jonathan Wilbrink for support with the fluorescence measurements. The authors further thank Marc Ankone for the support in the synthesis and Maaike Schotman and Regine van der Hee for the support in cell experiments. Furthermore, the team of Wyatt Technology Europe GmbH is thanked for the MALS analysis.

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