Core/shell protein-reactive nanogels via a combination of RAFT polymerization and vinyl sulfone postmodification

Aim: A promising nanogel vaccine platform was expanded toward antigen conjugation.

Materials & methods: Block copolymers containing a reactive ester solvophobic block and a PEG-like solvophilic block were synthesized via reversible addition-fragmentation chain-transfer polymerization. Following self-assembly in DMSO, the esters allow for core-crosslinking and hydrophilization by amide bond formation with primary amines. Free thiols were accessed at the polymer chain ends through aminolysis of the reversible addition-fragmentation chain-transfer groups, and into the nanogel core by reactive ester conversion with cysteamine. Subsequently, free thiols were converted into vinyl sulfone moieties. **Results:** Despite sterical constraints, nanogel-associated vinyl sulfone moieties remained well accessible for cysteins to enforce protein conjugation successfully. **Conclusion:** Our present findings provide a next step toward well-defined vaccine nanoparticles that can co-deliver antigen and a molecular adjuvant.

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Keywords: antigen conjugate • end-group modification • nanogels • RAFT polymerization • reactive ester

Formulating protein antigens as nanoparticles is emerging as a promising strategy to modulate and increase the adaptive antigen-specific CD8⁺ T-cell response. This occurs through stimulation of antigen crosspresentation by dendritic cells to CD8 T cells [1–5] and enhanced lymphatic transportation of nanoparticulate species relative to soluble ones [6–8]. Antigen crosspresentation and further downstream, the type, magnitude and durability of the adaptive immune response, can be further improved by co-delivery of immune-modulators [9,10]. Agonists of Toll-like receptors (TLRs) are among the most potent activators of the innate immune response identified to date and thus are under intensive investigation as adjuvants for vaccination [11] or as agents for anticancer immunotherapy [12]. Especially promising in this context are TLR7/8 agonists that trigger

endosomal danger receptors that typically recognize single-stranded RNAs generated during viral infection [13] but can also be triggered by a synthetic class of small molecule imidazoquinolines and guanosines [14].

Recently, we reported on pH-degradable self-assembled nanogels with a small molecule imidazoquinoline-based TLR7/8 agonist covalently ligated into the nanogel core [15]. Whereas soluble TLR7/8 agonist caused a systemic inflammatory response in mice upon subcutaneous administration, nanogel-ligated TLR7 agonist restricted the *in vivo* immune-activation to the draining lymph nodes while alleviating systemic inflammation [15–17]. This unique feature also promoted antigen-specific humoral and cellular immune responses against an admixed protein antigen. Degradable polymer nanogels were prepared by selective crosslinking of self-assembled amphiphi-

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lic reactive ester block copolymers. They were composed of a hydrophilic, poly(ethyleneglycol) (PEG)-like polymer block based on methoxytriethylene glycol methacrylate (mTEGMA) and a hydrophobic polymer block based on pentafluorophenyl methacrylate (PFPMA) that can be block copolymerized by reversible addition fragmentation chain-transfer (RAFT) polymerization [18–20]. The PEG-like hydrophilic block was used to provide nanoparticle stability and tissue mobility as recently demonstrated by us and others [6,9,21]. The PFPMA block allowed for self-assembly into nanoparticles in polar aprotic solvents (such as DMSO) followed by functionalization and crosslinking of the PFP esters with bisamines (containing a degradable linker) [22] without facing competing hydrolysis reactions that would occur in aqueous medium [23]. Furthermore, remaining PFP esters could be converted into hydrophilic moieties to yield fully hydrated nanogels after transfer to aqueous medium. In addition, we also recently demonstrated the use of this approach to engineer nanogels with dendritic cell-targeting properties by replacing the mTEGMA block by a mannosylated glycopolymer block [24].

In this work we aim at further engineering this nanogel platform by introducing protein-reactive moieties to enable antigen conjugation. Earlier, we have endeavored to use maleimide- and pyridyldisulfide-functionalized RAFT chain-transfer agents to generate protein-reactive polymers forming thioether and disulfide bonds with cysteine residues [25]. However, maleimides are instable in the presence of primary amines used for nanogel core crosslinking, and pyridyldisulfides are prone to disulfide exchange *in vivo* with randomly encountered cysteines leading to deconjugation. Moreover, there is recent evidence on the limited stability of maleimide-generated thioethers *in vivo* [26]. Therefore, in the present work, we elaborate on an alternative approach comprising post-polymerization modification of the thiocarbonyl end-group of RAFT polymers into a vinyl sulfone (VS) moiety that can react with free cysteine moieties by Michael-type addition, affording stable thioether bonds (Figure 1) [27]. In this approach, postpolymerization the thiocarbonyl moiety of the RAFT chain transfer agent (CTA) is reduced via aminolysis followed by reacting the resulting free thiol with a divinyl sulfone. Here we apply this chemistry to yield both shell and core VS-functionalized nanogels and evaluate the conjugation efficiency to bovine serum albumin (BSA) as model protein antigen.

Materials & methods

Materials

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich and Acros Organics.

2,2'-azobis(2-methylpropionitrile) (AIBN) as initiator was provided by Wako Chemicals and purified by recrystallization from diethyl ether prior to use. RAFT CTA 4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CETPA) and 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPTPA) and the monomers tri(ethylene glycol) methyl ether methacrylate (mTEGMA) and PFPMA were synthesized according to established procedures [22,28]. Amicon® Ultra-15 centrifugal filters with a MWCO of 3 or 10 kDa were obtained from Merck Millipore. Spectra/Por 3 dialysis membranes with an MWCO of 1 kDa were obtained from Spectrum Labs. For transmission electron microscopy (TEM) sample staining, D(+)-trehalose dihydrate (Fisher BioReagent) and uranyl acetate dihydrate (Riedel de Haën) were used. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Any kD™ Mini-PROTEAN® TGX™ precast gels from Bio-Rad.

Instrumentation

All ¹H-, ¹³C- and ¹⁹F-NMR spectra were recorded on a Bruker 300 MHz or 400 MHz FT NMR spectrometer. Chemical shifts (δ) are provided in ppm relative to tetramethylsilane (TMS). Samples were prepared in given deuterated solvents and their signals referenced to residual nondeuterated signals of the solvent. Molecular weight determination of the precursor polymers was obtained using size exclusion chromatography (SEC) in tetrahydrofuran (THF) as solvent. This system consisted of a PU 1580 pump, AS 1555 auto sampler, UV 1575 UV-detector (detection at 254 nm), RI 1530 RI-detector from JASCO (Groß-Umstadt, Germany). Columns were purchased at MZ-Analysentechnik: MZ-Gel SDplus 102 Å and MZ-Gel SDplus 106 Å. Calibration was done using poly(styrene) standards purchased from PSS Polymer Standard Services (Mainz, Germany). Ultraviolet-visible (UV-VIS) spectrophotometry was performed on a Biomate 5 Thermo Spectronic spectrometer. Elemental analyses were conducted by the Microanalytical Laboratory at the Institute of Organic Chemistry, Johannes Gutenberg-University Mainz (Germany). Dynamic light scattering (DLS) analyses were performed on a Zetasizer Nano S (Malvern Instruments Ltd, Malvern, UK) equipped with a HeNe laser (λ = 633 nm) and detection a scattering angle of 173°. All samples were filtered through Whatman nylon syringe filters (0.45 µm pore size, GE Healthcare Life Science) prior to each measurement. For TEM, an FEI Tecnai F20 transmission electron microscope was used operating at 200 kV and images were collected on a 2k CCD camera (Gatan Ultrascan 1000). For sample preparation, 10 µl of 1 mg/ml nanogel sample in water were applied

Figure 1. Protein conjugation of vinyl sulfone functionalized polymers or nanogels to free cysteine moieties in protein by Michael-type addition.

on a lacey carbon film mounted on a copper grid. Ten microliter of a negative staining solution (4% uranyl acetate/ 1% trehalose) was added and blotted after 5 min by a filter paper. Note that the usual negative contrast produced by uranyl acetate for nanomaterials is inverted in this case because the nanogel particles tend to incorporate the uranyl ion, thus, stain the nanoparticles themselves instead of the environment. Additionally, trehalose preserves the overall morphology of the nanomaterials [29].

Synthesis of pPFMA₃₃ using CPTA

In analogy to earlier reports [22], a Schlenk tube equipped with a stir bar was loaded with PFPMA (2.0 g; 7.932 mmol), CPTA (88.5 mg; 0.317 mmol) and AIBN (5.2 mg; 0.032 mmol). All compounds were dissolved in anhydrous dioxane (2.0 ml). Following three freeze-pump-thaw cycles the tube was immersed in an oil bath at 70 °C for 6 h under vigorously stirring. A ¹H-NMR sample of the reaction mixture dissolved in CDCl₃ was analyzed showing 50% monomer conversion. The resulting polymer was isolated by precipitation in hexane and centrifugation (the supernatant was slightly red from unreacted CTA). After re-dissolving in a few ml of dioxane, this process was repeated three times. The precipitated polymer was dried for 12 h at 40 °C under 10 mbar vacuum affording pPFMA₃₃ (determined by ¹H-NMR) (889 mg; 82%) as a red powder.

SEC (THF, PS-Std.): Number-average molecular weight (M_n) = 8,400 g/mol; Weight-average molecular weight (M_w) = 10,700 g/mol; Molecular weight dispersity ($\bar{D}$) = 1.29.

¹H-NMR (CDCl₃, 300 MHz): δ [ppm] = 8.20 – 7.30 (m, 5H, -C₆H₅ CTA phenyl end group); 3.05 – 1.90 (br, 2H, -CH₂- PFPMA polymer main chain);

1.90 – 1.20 (br, 3H, -CH₃ PFPMA polymer main chain).

Synthesis of pPFMA₄₆ using CETPA

In analogy to earlier reports [22], a Schlenk tube equipped with a stir bar was loaded with PFPMA (2.0 g; 7.932 mmol), CETPA (83.6 mg; 0.317 mmol) and AIBN (5.2 mg; 0.032 mmol). All compounds were dissolved in anhydrous dioxane (2.0 ml). Following three freeze-pump-thaw cycles the tube was immersed in an oil bath at 70 °C for 6 h under vigorously stirring. A ¹H-NMR sample of the reaction mixture dissolved in CDCl₃ was analyzed showing 52% monomer conversion. The resulting polymer was isolated by precipitation in hexane and centrifugation (the supernatant was slightly yellow from unreacted CTA). After re-dissolving in few ml of dioxane, this process was repeated three times. The precipitated polymer was dried for 12 h at 40 °C under 10 mbar vacuum affording pPFMA₄₆ (determined by ¹H-NMR) (907 mg; 81%) as a yellow powder.

SEC (THF, PS-Std.): M_n = 11,800 g/mol; M_w = 15,300 g/mol; $\bar{D}$ = 1.29.

¹H-NMR (CDCl₃, 300 MHz): δ [ppm] = 3.60 – 3.05 (m, 2H, -S-CS-S-CH₂- CTA ethyl end group); 3.05 – 1.90 (br, 2H, -CH₂- PFPMA polymer main chain); 1.90 – 1.20 (br, 3H, -CH₃ PFPMA polymer main chain).

Synthesis of CPTA pPFMA₃₃-b-pMTEGMA₃₅ (1)

In analogy to earlier reports [22], a Schlenk tube equipped with a stir bar was loaded with mTEGMA (760 mg; 3.27 mmol), pPFMA₃₃ (425 mg; 0.049 mmol) and AIBN (1.1 mg; 0.07 mmol). All compounds were dissolved in anhydrous dioxane (2.34 ml). Following three freeze-pump-thaw cycles the tube was

Table 1. Yields for various polymer and nanogel samples.

Yield	Polymer_1	Polymer_2	Nanogel_1	Nanogel_2
SH_CE	6.70 mg (72%)	8.84 mg (93%)	8.67 mg (62%)	15.39 mg (92%)
VS_CE	21.96 mg (85%)	27.33 mg (98%)	27.35 mg (68%)	23.71 mg (65%)
SH_BB+CE	8.44 mg (84%)	12.15 mg (97%)	8.10 mg (57%)	8.83 mg (58%)
VS_BB+CE	25.06 mg (90%)	37.29 mg (99%)	30.80 mg (70%)	39.28 mg (89%)

Figure 2. Design strategy for protein-reactive nanogels (see facing page). RAFT block copolymerization of PFPMA and mTEGMA was performed using CPTPA (R1) or CETPA (R2) as CTAs. After self-assembly in DMSO, the block copolymer micelles were crosslinked by addition 2,2'-(ethylenedioxy)bis(ethylamine). The residual PFP esters were quenched with 2-ethanolamine, which also reacts with the thiocarbonyl group producing free thiols to allow CE functionalization. Alternatively, after crosslinking, the remaining PFP esters were treated with cysteamine to allow for backbone functionalization prior to quenching with 2-ethanolamine BB. Free thiols were converted into pyridyldisulfide moieties, followed by transformation into vinyl sulfone.

BB: Backbone; CE: Chain end; CETPA: 4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid; CPTPA: 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid; CTA: Chain transfer agent;

mTEGMA: Methoxytriethylene glycol methacrylate; PFP esters: Pentafluorophenyl esters;

PFPMA: Pentafluorophenyl methacrylate; RAFT: Reversible addition-fragmentation chain-transfer.

immersed in an oil bath at 70°C for 18 h under vigorously stirring. A $^1\text{H-NMR}$ sample of the reaction mixture dissolved in CDCl_3 was analyzed showing 72% monomer conversion. The resulting polymer was isolated by precipitation in hexane and centrifugation. After redissolving in few ml of dioxane, this process was repeated three times. The precipitated polymer was dried for 12 h at 40°C under 10 mbar vacuum affording pPFPMA₃₃-b-pMTEGMA₃₅ (determined by $^1\text{H-NMR}$) (847 mg; 87%) as a slightly red powder.

SEC (THF, PS-Std.): $M_n = 11,500$ g/mol; $M_w = 14,000$ g/mol; $\text{Đ} = 1.22$.

$^1\text{H-NMR}$ (CDCl_3 , 300 MHz): δ [ppm] = 8.20 – 7.30 (m, 5H, $-\text{C}_6\text{H}_5$ CTA phenyl end group); 4.09 (br, 2H, $\text{COO}-\text{CH}_2-$); 3.65 (br, 8H, $\text{COO}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-$); 3.55 (br, 2H, $-\text{CH}_2-\text{O}-\text{CH}_3$); 3.38 (br, 3H, $-\text{O}-\text{CH}_3$); 2.85 – 2.10 (br, 2H, $-\text{CH}_2-$ PFPMA polymer main chain); 2.10 – 1.70 (br, 2H, $-\text{CH}_2-$ MEO₃MA polymer main chain); 1.70 – 1.20 (br, 3H, $-\text{CH}_3$ PFPMA polymer main chain) and 1.20 – 0.60 (br, 3H, $-\text{CH}_3$ MEO₃MA polymer main chain).

$^{19}\text{F-NMR}$ (CDCl_3 , 282 MHz): δ [ppm] = -150.41 – -151.52 (br, 2F, o-ArF); -157.00 (br, 1F, p-ArF); 161.96 (br, 2F, m-ArF).

Synthesis of CETPA pPFPMA₄₆-b-pMTEGMA₅₇ (2)

In analogy to earlier reports [22], a Schlenk tube equipped with a stir bar was loaded with mTEGMA (745 mg; 3.21 mmol), pPFPMA₄₆ (417 mg; 0.035 mmol) and AIBN (1.0 mg; 0.006 mmol). All compounds were dissolved in anhydrous dioxane (2.30 ml). Following three freeze-pump-thaw cycles the tube was immersed in an oil bath at 70°C for 18 h under vigorously stirring. A $^1\text{H-NMR}$ sample of the reaction mixture dissolved in CDCl_3 was analyzed showing 83% monomer conversion. The resulting polymer was isolated by precipitation in hexane and centrifugation. After re-dissolving in few ml of dioxane, this process was repeated three times. The precipitated polymer was dried for 12 h at 40°C under 10 mbar vacuum affording pPFPMA₄₆-b-pMTEGMA₅₇ (determined by $^1\text{H-NMR}$) (865 mg; 84%) as a slightly red powder.

SEC (THF, PS-Std.): $M_n = 19,800$ g/mol; $M_w = 24,000$ g/mol; $\text{Đ} = 1.21$.

$^1\text{H-NMR}$ (CDCl_3 , 300 MHz): δ [ppm] = 4.08 (br, 2H, $\text{COO}-\text{CH}_2-$); 3.65 (br, 8H, $\text{COO}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-$); 3.60 – 3.05 (m, 2H, $-\text{S}-\text{CS}-\text{S}-\text{CH}_2-$ CTA ethyl end group); 3.55 (br, 2H, $-\text{CH}_2-\text{O}-\text{CH}_3$); 3.38 (br, 3H, $-\text{O}-\text{CH}_3$); 2.85 – 2.05 (br, 2H, $-\text{CH}_2-$ PFPMA polymer main chain); 2.05 – 1.70 (br, 2H, $-\text{CH}_2-$ MEO₃MA polymer main chain); 1.70 – 1.20 (br, 3H, $-\text{CH}_3$ PFPMA polymer main chain) and 1.20 – 0.60 (br, 3H, $-\text{CH}_3$ MEO₃MA polymer main chain).

$^{19}\text{F-NMR}$ (CDCl_3 , 282 MHz): δ [ppm] = -151.21 – -152.21 (br, 2F, o-ArF); -157.85 (br, 1F, p-ArF); 162.94 (br, 2F, m-ArF).

Nanogel synthesis

Crosslinking

A detailed example of a one-pot nanogel synthesis using CPTPA pPFPMA₃₃-b-pMTEGMA₃₅ (1) is provided below. Yields for all synthesis products are given in Table 1. The precursor block copolymer 1 (150 mg; 296 μmol of reactive esters; 1 eq.) was transferred into a round-bottom flask equipped with a stirring bar and dispersed at 10 mg/ml in anhydrous DMSO (15 ml) under argon atmosphere. After sonication for 2 h, the resulting micellar dispersion was characterized by DLS. Crosslinking was performed by addition of 2,2'-(ethylenedioxy)bis(ethylamine) (10.8 μl , 74 μmol , 0.25 eq.) and triethylamine (TEA) (82.6 μl , 592 μmol , 2 eq.) and stirring 18 h at 50°C. This step was omitted to yield soluble control polymers with chain end (*Polymer_1*^{VS-CE}) or backbone (*Polymer_2*^{VS-CE+BB}) VS modification.

Chain end functionalization

Half of the crosslinked nanogel dispersions (75 mg; 4.5 μmol of CTA moieties, 148 μmol of reactive esters, 1 eq.) was used for VS shell functionalization. To guarantee complete removal of remaining PFP esters in the nanogel, the reaction mixture was quenched with excess 2-aminoethanol (26.8 μl ; 445 μmol ; 3 eq. relative to reactive esters) and TEA (186 μl , 1.33 mmol, 9 eq. relative to reactive esters). After 6 h of stirring at 50°C under argon atmosphere, an excess of 2,2'-dipyridyldisulfide (99 mg, 449 μmol , 100 eq. relative to

Table 2. Results of reversible addition-fragmentation chain-transfer homo and block copolymerization of pentafluorophenyl methacrylate and methoxytriethylene glycol methacrylate.

	CTA	[M] [CTA]: [AIBN]:	t (h)	p (%)	DP (NMR)	M _{NMR} (g/mol)	M _n (g/mol)	M _w (g/mol)	Đ
pPFPMA ₃₃	CPTPA	25:1:0.1	6	50	33	8600	8400	10,700	1.29
pPFPMA ₄₆	CETPA	25:1:0.1	6	52	46	11,900	11,800	15,300	1.29
pPFPMA ₃₃ -b-pMTEGMA ₃₅	pPFPMA ₃₃	65:1:0.15	18	72	35	16,700	11,500	14,000	1.22
pPFPMA ₄₆ -b-pMTEGMA ₅₇	pPFPMA ₄₆	90:1:0.15	18	83	57	25,100	19,800	24,000	1.21

AIBN: 2,2'-azobis(2-methylpropionitrile); CETPA: 4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid; CPTPA: 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid; CTA: Chain transfer agent.

CTA moieties) was added to the reaction mixture to cap the free thiols generated by aminolysis of the CTA moieties. After 14-h reaction at 50°C, the solution was diluted 10× with deionized water and purified five times by spin filtration with a 40:60 water:methanol mixture to remove the excess of 2-aminoethanol, TEA and 2,2'-dipyridyldisulfide. The purified sample was diluted 1:1 with a 0.1 M bicarbonate buffer pH 8.5 and analyzed by UV-vis spectroscopy. Subsequently the sample was added to an excess of tris(2-carboxyethyl) phosphine (TCEP, 26 mg, 89 μmol, 20 eq. relative to CTA moieties) and incubated for 15 min. The release of 2-pyridinethione upon cleavage of the disulfide linkages with the capped thiols was confirmed by UV-vis spectroscopy. An aliquot of this mixture was further purified by spin filtration without VS modification to yield non protein-reactive controls (*Nanogel-I^{SH-CE}*). The remaining mixture was added dropwise to an excess of divinyl sulfone (475 μl, 4.5 mmol, 1000 eq. relative to CTA moieties) and reacted for 14 h under argon atmosphere at room temperature. Finally, the reaction mixture was transferred into a dialysis mem-

brane and dialyzed against 50:50 water:methanol for 3 days, followed by dialysis against pure water and subsequent lyophilization (*Nanogel-I^{VS-CE}*).

Backbone functionalization

Half of the crosslinked nanogel dispersions (75 mg, 148 μmol of reactive esters, 1 eq.) was used for VS core functionalization by the introduction of free thiol groups on the reactive ester moieties. For that purpose, the crosslinked nanogels were reacted with cysteamine (8.4 mg, 74 μmol, 0.5 eq.) and TEA (82 μl, 592 μmol, 4 eq.) for 6 h under argon atmosphere at 50°C. To guarantee complete removal of remaining PFP esters in the nanogel, the reaction mixture was quenched with excess 2-aminoethanol (26.8 μl; 445 μmol; 3 eq.) and TEA (186 μl, 1.33 mmol, 9 eq.). In addition, an excess of 2,2'-dipyridyldisulfide (408 mg, 1.8 mmol, 12.5 eq.) was added to cap the free thiols originating from the introduced cysteamine moieties. After 14-h reaction at 50°C, the solution was purified by spin filtration and analyzed by UV-vis spectroscopy as described for the shell functionalized nanogels. Subsequently the sample

Figure 3. Size exclusion chromatography traces (eluent: tetrahydrofuran) of polypentafluorophenyl methacrylate homo and polypentafluorophenyl methacrylate-b-Polymethoxytriethylene glycol methacrylate block copolymers. pPFPMA: Polypentafluorophenyl methacrylate; PMTEGMA: Polymethoxytriethylene glycol methacrylate; SEC: Size exclusion chromatography; THF: Tetrahydrofuran.

Figure 4. Dynamic light scattering size distribution curves (volume mean diameter) of **1** and **2** measured at 10 mg/ml **(A)** in DMSO, **(B)** after chain end functionalization and **(C)** after backbone and chain end vinyl sulfone functionalization. Crosslinked nanogels (denoted as 'nanogel') and noncrosslinked controls (denoted as 'polymer') were measured in 0.1M bicarbonate buffer pH 8.5. DLS: Dynamic light scattering.

was added to an excess of TCEP (212 mg, 741 μmol , 5 eq.) and incubated for 15 min. Again the release of pyridyldisulfide upon cleavage of the disulfide linkages with the capped thiols was confirmed by UV-vis spectroscopy. An aliquot of this mixture was further purified by spin filtration without VS modification to yield non protein-reactive controls (*Nanogel_1^{SH_CE+BB}*). The remaining mixture was then added dropwise to an excess of divinyl sulfone (784 μl , 7.4 mmol, 50 eq.) and reacted for 14 h under argon atmosphere at room temperature. Finally, the reaction mixture was transferred into a dialysis membrane and dialyzed against 50:50 water:methanol for 3 days, followed by dialysis against pure water and subsequent lyophilization (*Nanogel_1^{VS_CE+BB}*).

Conjugation of VS-functionalized nanogels to BSA

An example conjugation procedure is described as follows. Stock solutions of BSA (1.16×10^{-4} M, 7.7 mg/ml), TCEP (0.1 M, 30 mg/ml) and nanogels (20 mg/ml) were prepared in a bicarbonate buffer (0.1 M, pH 8.2). The TCEP stock was combined with the BSA solution in a 1:10 ratio and allowed to react for 15 min. Subsequently, the BSA and polymer stock solutions were

combined to obtain a molar ratio of protein:polymer 1:40. Then the reaction mixture was diluted with buffer solution to obtain a protein concentration of 1.51×10^{-5} M. Next, 10% of DMSO was added and the mixture was incubated overnight at 20°C in a thermoshaker. Conjugation efficiency was evaluated by reducing SDS-PAGE using β -mercaptoethanol as a reducing agent. Quantification of protein conjugation was done by automated integration of optical density by ImageJ software and calculating the ratio of bound protein to total protein content per lane as described in previous work [25].

Results & discussion

The design strategy for protein-reactive nanogels is depicted in Figure 2. In a first step, block copolymers composed of poly(PFPMA) and poly(mTEGMA) were prepared via RAFT polymerization using, respectively, CPTPA or CETPA as a CTA and AIBN as radical initiator. We started with polymerizing PFPMA affording pPFPMA polymers with either a dithiobenzoate end group (pPFPMA₃₃ generated by CPTPA) or an ethyl trithiocarbonate end group (pPFPMA₄₆ generated by CETPA) that could both be used as a macroCTA for the polymerization of mTEGMA. The reason for choosing

Figure 5. UV-VIS spectroscopy analysis of the nanogels before (blue curves) and after (red curves) the addition of tris(2-carboxyethyl)phosphine demonstrates the release of pyridine-2-thione ($\lambda_{\text{max}} = 343 \text{ nm}$) upon reduction of disulfide linkages, thereby generating free thiols that can subsequently be modified with divinyl sulfone. CEP: Tris(2-carboxyethyl)phosphine; UV-VIS: Ultraviolet-visible.

this sequence, that is, first polymerizing PFPMA and then mTEGMA, is because we desired to obtain block copolymers with the thiocarbonyl RAFT end group at the hydrophilic chain end for further postmodification into a cysteine-reactive VS moiety. Table 2 summarizes the properties of the respective syntheses of well-defined block copolymers pPFPMA₃₃-*b*-pMTEGMA₃₅ and pPFPMA₄₆-*b*-pMTEGMA₅₇, further denoted as 1 and 2. SEC elugrams recorded using THF as eluent are shown in Figure 3, and ¹H- and ¹⁹F-NMR spectra can be found in the Supplementary section (Supplementary Figures 1–6).

In DMSO, both block copolymers 1 and 2 formed self-assembled nanoparticles with a mean diameter of, respectively, 25 and 40 nm, as measured by DLS (Figure 4A & Supplementary Figures 9 & 10A). Next, the self-assembled nanoparticles were core-crosslinked by addition of 0.25 equivalents 2,2'-(ethylenedioxy) bis(ethylamine) with respect to reactive esters, forming stable intra- and interpolymer amide bonds inside the core. For the sake of simplicity, we elaborated in this work solely on nondegradable nanogels. However, we and others have previously shown that this

class of nanogels can easily be rendered degradable in response to endosomal pH using ketal-based bisamine crosslinkers [15,23]. As controls, we also prepared soluble polymers – instead of nanogels – by omitting this crosslinking step, which will further on in this report be denoted as ‘polymer’, whereas crosslinked nanogels will be denoted as ‘nanogel’.

Subsequently, the crosslinked nanoparticles were further engineered with cysteine-reactive moieties at either the poly(mTEGMA) chain ends or both the poly(mTEGMA) chain ends and the backbone of the inner-core polymers. For engineering cysteine-reactivity into the nanoparticles only at the chain-ends, after the crosslinking step, all remaining PFP-esters were converted by an excess of 2-aminoethanol. The use of 2-aminoethanol renders the inner core of the nanoparticles hydrophilic and yields fully hydrated nanogels upon transfer of the nanoparticles from DMSO to aqueous medium. To introduce cysteine-reactive moieties into the backbone of the inner core polymers, after crosslinking the nanoparticles were first treated with 0.5 equivalents of cysteamine to convert remaining PFP-esters into mercaptoethyl methacrylamide

moieties. Subsequently, the remaining unreacted PFP-esters were also converted to hydrophilic repeating units by addition of an excess 2-aminoethanol. For the sake of clarity, vinylsulfone-functionalized nanogels and polymers will be annotated with ^{VS}, with ^{CE} referring to nanogels with VS groups only at the poly(mTEGMA) chain ends and ^{CE+BB} referring to nanogels with VS groups both at the poly(mTEGMA) chain ends and the backbone of the inner core polymer chains.

In the presence of primary amines, aminolysis of the thiocarbonyl RAFT end-groups occurs and yields free thiols at the poly(mTEGMA) chain ends. This process provides direct access to a functional polymer chain end and omits the use of modified chain transfer agents for RAFT polymerization, which would have to be synthesized prior to polymerization [30]. In our case, the used RAFT CTAs (CPTPA and CETPA) afforded block copolymers with either a dithiobenzoate or ethyl trithiocarbonate end group that are both suitable for conversion into free thiols by aminolysis. These chain end moieties as well as the thiols introduced into the nanogel core via cysteamine, were subsequently converted into cysteine-reactive VS moieties. For this purpose, the nanoparticles were transferred from DMSO to water via dialysis which also removes low molecular weight byproducts and unreacted species. Next, we attempted to react the free thiols with an excess of divinyl sulfone via Michael-type thiol-ene reaction in presence of TCEP to reduce possibly formed disulfides. However, we observed that over the time course of several days during dialysis the basic milieu that was generated for PFP-ester and RAFT end group aminolysis triggers extensive oxidation of the free thiols, thereby preventing subsequent Michael-type reaction with divinyl sulfone. Ellman's assay on these samples confirmed the absence of free thiols (Supplementary Figure 7). To circumvent this issue, immediately after PFP-ester conversion, free thiols were capped by addition of 2,2'-dipyridyldisulfide in a one pot setting without the need for intermitted purification [31]. Besides avoiding oxidation during further work-up, capping free thiols with 2,2'-dipyridyldisulfide also avoided unwanted interparticle crosslinking by disulfide formation [32]. After removal of low molecular weight byproducts and the excess of unreacted species by spin filtration, free thiols were regenerated upon reduction of the disulfide linkages by TCEP, as monitored by UV-VIS spectrophotometric detection of the released pyridine-2-thione (Figure 5 for nanogels, Supplementary Figure 8 for polymers). Note that spin filtration was preferred over dialysis to shorten the time scale of preparation and reduce the risk of unwanted oxidative side reactions. Subse-

Figure 6. Transmission electron microscopy images of the various types of core-crosslinked nanogels (scale bar 0.2 μ m, staining by uranyl acetate).

TEM: Transmission electron microscopy.

quently, the free thiols were reacted with an excess of divinyl sulfone to yield cysteine-reactive VS functionalized nanogels. Additionally, we also prepared control nanogels and soluble polymers by omitting the divinyl sulfone modification step. Note that in this case, due to the TCEP treatment, all pyridyldisulfide moieties at the polymer chain ends and backbone were converted into free thiols (SH). Finally, all samples were extensively dialyzed and obtained as a dry powder after lyophilization.

DLS analysis of redispersed samples in aqueous medium are shown in Figure 4B & C and in the Supplementary Figures 9, & 10B & C. For both 1 and 2, crosslinking of the self-assembled micellar structures in DMSO (*vide supra*) with bisamines afforded the formation of nanogels with similar sizes in aqueous medium as those measured in DMSO before transformation of the PFP esters (Figure 4A). An overlay of the intensity size distribution plots for each different nanogel species (Supplementary Figure 9) confirmed these observations. No difference was found for either chain end VS functionalization or backbone and chain end functionalization. In addition, by TEM particles were visualized for all core-crosslinked nanogel species within the size regime detected by DLS (Figure 6). Interestingly, when the crosslinking step was omitted, chain end VS functionalized polymers 1^{VS_CE} and 2^{VS_CE} were fully water soluble due to the introduction of 2-aminoethanol onto the reactive ester moieties and DLS indicated unimers of about 5 nm and 6.6 nm for 1 and 2, respectively. However, backbone functionalization of the polymers (i.e., 1^{VS_CE+BB} and 2^{VS_CE+BB})

Figure 7. ^1H -NMR (400 MHz, CD_3OD) δ (ppm) of noncrosslinked polymer 1 and 2 with chain end ($^{\text{VS_CE}}$) and backbone ($^{\text{VS_BB}}$) vinyl sulfone functionalization (see facing page).

with VS-introduced new hydrophobic moieties onto the reactive ester moieties and caused some ill-defined aggregate formation, in particular in case of $2^{\text{VS_CE+BB}}$. The DLS correlograms of these aggregates compared with the well-defined nanogel species are given in the **Supplementary Figure 10B & C**.

To confirm successful incorporation of VS, NMR analysis was performed on the noncrosslinked polymers (**Figure 7**). For the chain end functionalized polymer ($^{\text{VS_CE}}$) we can clearly distinguish the VS proton peaks at 6.25–6.50 ppm and 6.85–7 ppm, respectively, but they are relatively weak as expected

Figure 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results from the conjugation of bovine serum albumin (66 kDa) with chain end (CE) and backbone (CE+BB) vinyl sulfone functionalized (VS) noncrosslinked polymers P1 and P2 in a molar ratio of protein:polymer 1:40 and 1:80. Additionally, nonfunctionalized polymers ($^{\text{SH}}$) were used as negative control (mean $\pm$ SD, $n = 3$). BSA: Bovine serum albumin; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Figure 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results from the conjugation of bovine serum albumin (66 kDa) with chain end (CE) and backbone (CE+BB) vinyl sulfone functionalized (VS) crosslinked nanogels N1 and N2 in a molar ratio of protein:nanogel 1:40 and 1:80. Additionally, nonfunctionalized nanogels (SH) were used as negative control (mean $\pm$ SD, $n = 3$).

BSA: Bovine serum albumin; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

since only one VS per polymer is possible, compared with the side chain functionalized VS (VS_{CE+BB}) where broader peaks with an increased intensity due to multiple VS per polymer were observed. However, in both cases peak integration was not accurate enough for quantitative analysis. In addition, elemental analysis was performed for the core-crosslinked nanogels, since NMR analysis is less favorable due to restricted diffusive mobility inside the nanogel network. In [Supplementary Table 2](#), the sulfur content found for both chain end and backbone incorporation modified nanogels was – within the accuracy of the detection method – in accordance to calculated values based on [Supplementary Table 1](#). These results confirm VS

modification of the aminolyzed chain ends and the cysteamine converted backbone.

Finally, we assessed the conjugation efficiency of the VS functionalized nanogels and soluble control polymers. For this purpose, we used BSA as model protein, and conjugation was assessed by SDS-PAGE gel electrophoresis under reducing conditions in presence of β -mercaptoethanol. The reason for using BSA is its commercial availability and widespread use as model protein. BSA contains a cysteine that does not participate in a disulfide bond (Cys-34). However, this thiol can be partially oxidized and therefore BSA was pretreated with 10 mM TCEP. We have previously demonstrated that this mild reductive treatment

induces cleavage of one disulfide bond, exposing two additional free thiols [33]. The subsequent conjugations were performed in a 0.1 M bicarbonate buffer at pH 8.5 containing 10% DMSO to enhance solubility of the VS moieties. Either 40 or 80 equivalents of nanogel/polymer to protein were added. In a first series of conjugation experiments, we used the noncrosslinked soluble polymers, with both chain end and chain end plus backbone VS modification, to establish a proof-of-concept for VS-based conjugation for this type of polymers.

The SDS-PAGE data in Figure 8 clearly demonstrates successful conjugation of the VS-modified polymers to BSA. Note that SDS-PAGE was run under reducing conditions in presence of β -mercaptoethanol, which means that any disulfide formation between BSA and free thiols on the control polymers and nanogels were cleaved. Optical integration using the ImageJ software package was performed to obtain semiquantitative data on the fraction of conjugated protein, expressed as bound protein to total protein content per lane as described in previous work [25]. Not unexpectedly, a higher conjugation efficiency is obtained for polymers that contain VS moieties both at their chain end and backbone, that is, $polymer^{VS_CE+BB}$, as this offers multiple sites for protein conjugation per polymer chain. These $polymer^{VS_CE+BB}$ samples also yield higher molecular weight conjugates, evidenced by the emergence of a broad band on the SDS-PAGE gel close to where the samples were loaded. Likely, this is due to multiple BSA molecules that became crosslinked by one or more polymers. With regard to the effect of the polymer to protein ratio, no significant increase in the fraction of conjugated protein is observed. These findings also point out the relatively low binding efficiency of a 'grafting to' approach when attempting to conjugate relatively large polymers to relatively large proteins. In previous work, we have therefore already explored a 'grafting from' method as an alternative conjugation approach [34]. However, this drawback might be less pronounced for smaller, biologically relevant peptide antigens that are less sterically hindered, or for recombinant proteins that are engineered with readily accessible pending cysteine residues.

In a second series of experiments, BSA was conjugated to nanogels bearing VS moieties either at their chain ends or both their chain ends and inner core backbone. Interestingly, similar conjugation efficiencies were obtained for nanogels (Figure 9) as for the soluble control polymers. First of all, this means that sterical restriction induced from the crosslinking does not affect the ability of the chain end VS moieties to conjugate to proteins. Second, this also

means that VS moieties located on the backbone of the inner core polymer block are also accessible for protein conjugation. This can likely be attributed to the fully hydrated nature of the nanogels, which can allow inward diffusion of proteins or to a more *compound micelle* like assembly, which means that both polymer blocks are not fully phase separated, but also intermixed. Such structure would lead to a strong increase of cysteine-reactive VS moieties on the nanogel surface.

Conclusion

In summary, we have demonstrated an efficient strategy to engineer core-crosslinked polymer nanogels with cysteine-reactive VS moieties that allow for the preparation of protein-ligated nanogels. Polymer nanogels were obtained by self-assembly of amphiphilic block copolymers containing a solvophobic poly(pentafluorophenyl) block, which serves both as a scaffold for crosslinking as well as for the introduction of free thiols that are subsequently transformed into VS moieties. In addition, the unique feature of RAFT polymerization that readily yields access to thiol terminated polymers was also used to introduce VS moieties at the hydrophilic polymer chain ends. Importantly, when compared with noncrosslinked soluble control polymers, crosslinked nanogels exhibit similar conjugation efficiencies, pointing out that the installed VS moieties are well accessible for cysteine-conjugation, even to a relatively large protein as BSA (66.5 kDa). In view of our earlier findings that a TLR7/8 agonist ligated to the core of these nanogels allows for potent lymph node restricted immune activation, our present findings further pave the road for the design of engineered nanocarriers for vaccine delivery that can combine potent immune-stimulatory molecules with peptide or protein antigens to further amplify cellular and humoral immune responses.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2016-0214

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- In previous work, the employed nanogel system has proved its alluring properties in view of vaccine delivery by selective lymph node activation when conjugated with a Toll-like receptor ligand.
- In the present study, we equipped these nanogels with cysteine-reactive vinyl sulfone (VS) moieties to allow site-specific antigen conjugation.
- Following aminolysis of the reversible addition-fragmentation chain-transfer thiocarbonyl groups, VS moieties could be introduced at the chain end of well-defined block copolymers containing a reactive pentafluorophenyl ester block.
- Additionally, VS could be incorporated in the polymer backbone by reacting the pentafluorophenyl esters with cysteamine.
- Even when these polymers were core-crosslinked, the installed VS moieties remained accessible for antigen conjugation.
- This platform enables the design of vaccine nanocarriers that can combine immune-stimulatory molecules with protein antigens.

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