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Improved SiRNA Loading of Cationic Nanohydrogel Particles by Variation of Crosslinking Density

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This paper deals with a concept to improve the loading capability of cationic nanohydrogel particles with siRNA. For this purpose, a new tetrafunctional crosslinker is synthesized via a peptide coupling approach using lysine and spermine derivatives. Applying this four-arm crosslinker to the particle synthesis makes it possible to perform the crosslinking with an excess or a deficit of the crosslinker. This allows for varying numbers of cationic groups per cationic core and its crosslinking density while the stability of the carrier system remains. The obtained cationic nanohydrogel particles are narrowly distributed in size as determined by dynamic light scattering measurements. Zeta potential measurements confirm the cationic nature of these carriers ($\zeta > +30$ mV) and the ability to form complex anionic siRNA (agarose gel electrophoresis). As a result, it becomes possible to increase the siRNA loading by a factor of four by varying the composition of the crosslinked core. Such an increased siRNA loading should lead to an improved therapeutic gene knockdown effect.

1. Introduction

Gene therapy has increasingly gained interest over the last few years to treat a great range of diseases. As a process in which foreign genomic material is introduced into a host cell and thereby a therapeutic effect can be achieved, gene therapy is intensively studied using plasmid DNA (pDNA), short interfering RNA (siRNA) or messenger RNA (mRNA).[1,2] SiRNA, as a short oligonucleotide, activates a mechanism called RNA interference (RNAi) when it enters a cell and thereby leads to a gene silencing.[3,4] But due to its polyanionic character, siRNA cannot reach its target side on its own. First, it will be degraded by RNases within the blood and second, as a highly charged molecule, it cannot pass the cell membrane and enter the cell. Thus, a carrier system is necessary to enable a safe and efficient delivery of siRNA.[5] Approaches for pDNA delivery are mostly based on polyplex systems. Such polyplexes are generated by polycationic polymers which can compact the large pDNA while stable carrier systems are obtained.[6,7] Since siRNA is much smaller than pDNA classical polyplex systems cannot stabilize this cargo efficiently. Thus, carrier systems are necessary which can be stabilized later on by crosslinking or which are already stable without the siRNA.[8] For this purposes, cationic nanohydrogel particles which were developed by our group can be used as efficient siRNA carriers.[9,10] These particles are prepared from amphiphilic reactive ester block copolymers, which are pre-assembled into micellar structures. Crosslinking the reactive ester core (hydrophobic) with suitable oligoamines in a polymer analogous reaction results in the formation of covalently crosslinked nanocarriers with a multi-cationic core, which can be loaded with negatively charged siRNA later on. Such cationic nanohydrogels are widely studied as siRNA carrier system. It has been shown that the gene silencing is clearly size-dependent, since particles with an average size of 40 nm or less induce a significant gene knockdown, while larger particles (above 100 nm) lack this effect.[11] They show a good siRNA stabilization capability within the bloodstream[12,13] and have already been used for the treatment of liver fibrosis.[14] Furthermore, it was possible to modify the hydrophilic corona of these particles by attaching mannose moieties, thereby achieving specific targeting of M2-polarized macrophages within the liver.[15]

Until now, all crosslinkers used for the synthesis of cationic nanohydrogel particles were bifunctional. The mostly evaluated crosslinker is the oligoamine spermine (see **Figure 1**A). The two primary amines react very efficiently with the reactive ester units in the polymer chains and are responsible for the crosslinking step, whereas the secondary amines are rather insensitive to aminolysis.[16,17] Thus, they do hardly participate in the crosslinking. However, they are protonated under physiological conditions, and thus, enable the complexation of negatively charged siRNA by electrostatic interactions. The use of spermine as first-generation crosslinker leads to particles which can complex siRNA sufficiently but also other crosslinkers, like a disulfide-containing crosslinker developed by Nuhn et al.[18] or a ketal-containing crosslinker synthesized by Leber et al.[19] have been studied. However, all these second-generation crosslinkers led to particles with decreased siRNA loading compared to the spermine particles. Thus, current studies focus on the improvement of the siRNA complexation capability of these nanohydrogels.

The ability to transport siRNA within these nanohydrogels is dependent both i) on the number of positive charges (amines)

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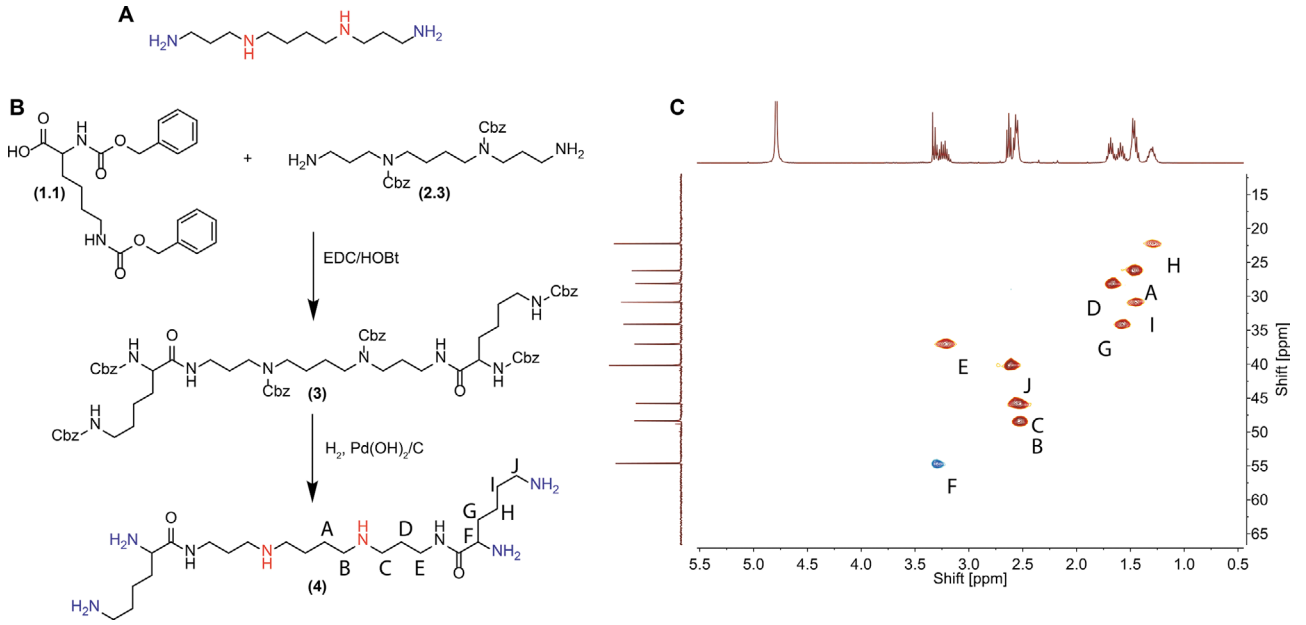

Figure 1. A) Chemical structure of two-arm crosslinker spermine (crosslinking amines in blue, cationic amines in red), B) synthesis of four-arm crosslinker (**4**) obtained by peptide coupling of Z,Z-lysine (**1.1**) and N4,N8-Z,Z-spermine (**2.3**) (crosslinking amines in blue, cationic amines in red), and C) corresponding HSQC (^{1}H and ^{13}C) NMR spectra of the final four-arm crosslinker (**4**).

within the crosslinkers (or more generally the final cationic core) and ii) on the crosslinking density, which determines how well siRNA gets access to the cationic core. Thus ideally a highly charged but only slightly crosslinked core with long polymer chains between the netpoints would be desirable.[20] This interferes, however, with the stability of the cationic nanohydrogels. During the introduction of cationic charges in the core of the micellar aggregates (which had been highly apolar before) this core becomes polar and water soluble and only a dense crosslinking keeps the nanostructure stable. Thus, only a quick and efficient crosslinking can stabilize the pre-assembled micellar nanostructure so that covalently stable nanohydrogels are accessible.

Thus, so far, all cationic nanohydrogel particles had been fully crosslinked (1 equiv. of crosslinker per 2 equiv. of reactive ester, or each repeating unit on the polymer chain was set as a netpoint to achieve 100% crosslinking density (CLD). In this context crosslinker units with a higher functionality of more than two primary amines get interesting, as they improve the connectivity of the polymer chains.

In this work, we present the development of a new four-arm amine crosslinker which can be used for the synthesis of cationic nanohydrogels. Stable particles with varying CLD were prepared using the new four-arm crosslinker and characterized. Furthermore, the impact of the CLD on the complexation ability of siRNA with these particles was evaluated.

2. Experimental Section

2.1. Materials

All chemicals were obtained from common commercial sources and were used as received, unless otherwise indicated. Anhydrous dimethyl sulfoxide (DMSO) was obtained from Acros and stored over molecular sieve (4 Å). Dichlormethane (DCM) was dried over calcium hydride (CaH_2) while dioxane was dried over sodium (Na). Both were subsequently distilled prior to use. *N,N*-diisopropylethylamine (DIPEA) was dried over NaOH and fractionally distilled in vacuo. 2,2′-Azobis(isoburyronitrile) (AIBN) was recrystallized from diethyl ether and stored at −18 °C. Oregon Green 488 cadaverine was obtained from Invitrogen. Phosphate buffered saline (PBS) was obtained from Fisher BioReagents containing 137 mM NaCl, 11.9 mM phosphate, and 2.7 mM KCl. Column chromatography was done using silica obtained from Machery-Nagel (0.063-0.2 mm/20-230 mesh). For dialysis, ZelluTrans membranes from Roth with a molecular weight cutoff of 6000–8000 g mol^{-1} were used.

2.2. Methods

^{1}H, ^{13}C, ^{19}F, DOSY, and HSQC spectra were recorded on a 400 and 100 MHz Bruker AvanceNII 400, respectively, and were referenced internally to residual proton signals of the deuterated solvent ($CDCl_3$: $\delta = 7.26$ ppm; DMSO-d_6: $\delta = 2.50$ ppm).[21]

Electron spray ionization mass spectrometry (ESI MS) was performed on an Agilent 6545 QTOF-MS with a concentration of 0.1–0.2 g L^{-1} in methanol (MeOH), MeCN, or water.

Dynamic light scattering (DLS) measurements were performed at 25 °C using a Malvern Zetasizer NanoZS with a 633 nm He/Ne Laser at a fixed scattering angle of 173°. Nanoparticles were measured at 0.1 g L^{-1} in PBS and filtered with GHP filters 0.2 µm pore size. For zeta potential measurements samples were measured at 1.0 g L^{-1} in milli pore water. Three independent measurements were performed and calculated as mean ± standard deviation.

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Agarose gel electrophoresis was performed using a 0.75 w% agarose gel prepared with TBE buffer and GelRed for DNA staining. The samples were prepared in PBS buffer at given weight-to-weight ratios and incubated at room temperature for 30 min. After addition of loading buffer, all samples were transferred onto the gel and analyzed at 120 V for 30 min. Visualization occurred in a dark hood under UV light (365 nm) and documentation was done by a DCC camera (Intas). Complexation experiments were performed with scrambled (random sequence) siRNA which was purchased from IBA GmbH (Göttingen, Germany) with the following sequence:

Sense strand: 5′- AGG UAG UGU AAU CGC CUU C TT-3′
Anti-sense strand: 3′- TT UUC AUC ACA UUA GCG GAA C-5′

To determine the complexation rate of siRNA in the nanoparticles NP1 to NP4 agarose gel electrophoresis was performed (see Figure 4). To determine the mass ratio of maximal loading the bands of free siRNA were evaluated with the software ImageJ. An example is shown in Figure S10, Supporting Information.

2.3. Syntheses

*2.3.1. N,N-(benzyloxy carbonyl)lysine (Z,Z-lysine, (**1.1**))*

The reaction was done as reported by Schweiker et al.[22]

^{1}H NMR (400 MHz, $CDCl_3$) δ [ppm] = 7.44 – 7.27 (m, 10H, aromatic-CH), 5.26 – 4.93 (m, 4H, benzylCH_2), 4.50 – 4.17 (m, 1H, α-CH), 3.27 – 2.99 (m, 2H, ε-CH_2), 1.99 – 1.59 (m, 2H, β-CH_2), 1.59 – 1.29 (m, 4H, γ,δ-CH_2).

*2.3.2. N1,N12-bis(trifluoroacetyl)spermine bistrifluoroacetate (N1,N12-TFA-spermine, (**2.1**))*

According to literature,[23] spermine (5.88 g, 29.1 mmol) was dissolved in MeCN (80 mL) and water (1.4 mL). Ethyl trifluoroacetate (TFA) (21.5 g, 151.3 mmol) was added and the reaction was refluxed overnight. After removal of the solvent under reduced pressure and washing with DCM the product was obtained as a pale yellow-colored solid (21.1 g, quantitative) and used without further purification.

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 9.61 (t, J = 5.8 Hz, 2H, TFA—NH); 8.75 (s, 4H, CH_2—NH_2^+—CH_2); 3.26 (q, J = 6.6 Hz, 4H, CH_2—NH—TFA); 2.92 (s, 8H, CH_2—NH_2^+—CH_2); 1.82 (p, J = 7.1 Hz, 4H, NH—$CH_2CH_2CH_2$—NH), 1.71 – 1.54 (m, 4H, NH—$CH_2CH_2CH_2CH_2$—NH).

*2.3.3. N1,N12-bis(trifluoroacetyl)-N4,N8-bis(benzyloxy carbonyl) spermine (N1,N12-TFA-N4,N8-Z,Z-spermine, (**2.2**))*

The reaction was done as previously reported.[24] N1,N12-TFA-spermine (21 g, 33.75 mmol) was dissolved in THF (75 mL) and a solution of K_2CO_3 (46.5 g) in water (45 mL) was added. Benzyl chloroformate (Cbz-Cl, 23 g, 19.2 mL, 135.3 mmol) was added over 30 min and the reaction continued for 24 h under vigorous stirring. Brine (100 mL) was added and the mixture was extracted with DCM. After drying the organic layers over $MgSO_4$ the solvent was removed under reduced pressure yielding the crude product. Column chromatography with DCM and 1% of methanol as eluent gave N1,N12-bis(trifluoroacetyl)-N4,N8-bis(benzyloxy carbonyl)spermine as pale yellow oil (16.8 g, 75% (Lit.[24] 98%), R_f = 0.2).

^{1}H NMR (400 MHz, $CDCl_3$) δ [ppm] = 8.08 (s, 1H, TFA—NH); 7.43 – 7.24 (m, 10H, aromatic-CH); 5.12 (s, 4H, benzyl—CH_2); 3.41 – 2.99 (m, 10H, CH_2—NH—CH_2); 1.67 (s, 4H, NH—$CH_2CH_2CH_2$—NH); 1.46 (s, 4H, NH—$CH_2CH_2CH_2C$ H_2—NH).

*2.3.4. N4,N8-bis(benzyloxycarbonyl)spermine (N4,N8-Z,Z-spermine, (**2.3**))*

The reaction was done according to the literature.[24] A solution of N1,N12-TFA-N4,N8-Z-spermine (16.8 g, 25.4 mmol) in MeOH (55 mL) and ammonium hydroxide (NH_4OH, 110 mL) was refluxed overnight under vigorous stirring. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography using methanol and 5% NH_4OH as eluent. N4,N8-bis(benzyloxycarbonyl)spermine was obtained as yellow oil (10.3 g, 86%, (Lit.[24] 91%) R_f = 0.25).

^{1}H NMR (400 MHz, $CDCl_3$) δ [ppm] = 7.42 – 7.27 (m, 10H, aromatic-CH); 5.19 – 5.00 (m, 4H, benzyl—CH_2); 3.40 – 3.05 (m, 8H —CH_2—NH—CH_2—); 2.75 – 2.54 (m, 4H, CH_2—NH_2); 1.72 – 1.57 (m, 4H, NH— CH_2—CH_2 CH_2—NH); 1.57 – 1.33 (m, 4H, NH—CH_2—CH_2—CH_2—CH_2—NH).

*2.3.5. Peptide Coupling Using Z,Z-lysine and N4,N8-Z,Z-spermine (**3**)*

Solution A Z,Z-Lysine (1 g, 2.41 mmol) was dissolved in anhydrous DCM (15 mL) and DIPEA (311.5 mg, 410 µL, 2.41 mmol) was added. EDC • HCl (462 mg, 241 mmol) and HOBt (2.77 mg, 205 mmol) were added and the reaction was stirred for 4 h under argon atmosphere.

Solution B N4,N8-Z,Z-spermine (567,1 mg, 1.21 mmol) was dissolved in anhydrous DCM (5 mL) and DIPEA (311.5 mg, 410 µL, 2.41 mmol). After adding solution B to A, stirring was continued for 20 h at room temperature and argon atmosphere. The reaction was extracted with water (15 mL) and brine (10 mL). After drying the organic layer with $MgSO_4$ and removal of the solvent under reduced pressure the crude product was obtained. Column chromatography with DCM/MeOH/NEt_3 (94:5:1) as eluent yielded the coupling product as colorless oil (1.05 g, 69%).

^{1}H NMR (400 MHz, $CDCl_3$) δ [ppm] = 7.68 – 7.02 (m, 30H, aromatic-CH), 5.22 – 4.86 (m, 12H, benzyl CH_2), 4.31 – 4.05 (m, 2H, α—CH_2), 3.34 – 2.98 (m, 12H, N—CH_2—), 2.94 – 2.34 (m, 4H, ε—CH_2), 1.96 – 1.07 (m, 20H, β,γ,δ—CH_2 + —CH_2—).

*2.3.6. N1,N12-bis(2,6-diaminohexanamido)spermine (four-arm crosslinker, (**4**))*

N4,N8-Z,Z-spermine (407 mg, 0.32 mmol) was dissolved in MeOH (7 mL). $Pd(OH)_2$/C (90.4 mg, 0.64 mmol) was added.

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Under constant shaking and hydrogen atmosphere the reaction was continued for 18 h. Filtration over cellite and removal of the solvent under reduced pressure yielded the multifunctional four-arm crosslinker as colorless oil (130 mg, 0.28 mmol, 88%).

^{1}H NMR (400 MHz, D_2O) δ [ppm] = 3.35 – 3.31 (m, 2H, α–CH_2), 3.30 – 3.15 (m, 4H, OC–NH–CH_2), 2.63 (t, J = 7.1 Hz, 4H, ε–CH_2), 2.60 – 2.50 (m, 8H, CH_2–NH–CH_2), 1.68 (p, J = 7.0 Hz, 4H, NH–CH_2–CH_2–CH_2–NH), 1.63 – 1.53 (m, 4H, β–CH_2), 1.53 – 1.39 (m, 8H, δ–CH_2+NH–CH_2–CH_2–CH_2–CH_2–NH), 1.39 – 1.21 (m, 4H, γ–CH_2).

^{13}C NMR (100 MHz, D_2O) δ [ppm] = 177.67 (carbonyl-C), 54.62 (α–CH_2), 48.34 (NH–CH_2–CH_2–CH_2–CH_2–NH), 45.78 (NH–CH_2–CH_2–CH_2–NHCO), 40.17 (ε–CH_2), 37.03 (NH–CH_2–CH_2–CH_2–NHCO), 34.11 (β–CH_2), 30.87 (δ–CH_2), 28.10 (NH–CH_2– CH_2–CH_2–NHCO), 26.23 (NH–CH_2–CH_2–CH_2–CH_2–NH), 22.25 (γ–CH_2).

ESI MS: m/z = 459.41 $[M+H]^+$, 481.39 $[M+Na]^+$ (calc. 458.41 g mol^{-1}).

2.3.7. Block Copolymer $P(MEO_3MA)_{24}$-b-$P(PFPMA)_{32}$

The synthesis of the amphiphilic block copolymer $P(MEO_3MA)_{24}$-*b*-$P(PFPMA)_{32}$ via RAFT polymerization was already reported.[19]

2.3.8. Cationic Nanohydrogel Particle Synthesis Using the Four-Arm Crosslinker

The synthesis of cationic nanohydrogel particles established by Nuhn et al.[10] crosslinked with the new four-arm moiety followed the general procedure.[9]

In a round bottom flask, the amphiphilic block copolymer $P(MEO_3MA)_{24}$-*b*-$P(PFPMA)_{32}$ (40 mg, 2.94 or 94.1 µmol PFPMA units) was dissolved in anhydrous DMSO (4 mL). Self-assembly of the polymer into micelles was supported by sonication for 1–2 h until a clear solution was obtained; afterward transferred into a Schlenk flask equipped with a stir bar and argon atmosphere. TEA (7.3 µL (≈10% of total 78.3 µL, 57.1 mg, 564.5 µmol) and Oregon Green 488 cadaverine (93.4 µL of a 2.5 g L^{-1} stock solution in DMSO, 0.470 µmol) were added and the reaction was stirred for 18 h at room temperature under exclusion of light. Then, the four-arm crosslinker (10.8 mg, 23.5 µmol for NP1 and 5.4 mg, 11.75 µmol for NP4) and TEA (71 µL (≈90% of total 78.3 µL, 57.1 mg, 564.5 µmol) were added and the orange solution was stirred for 18 h at 50 °C. Complete PFPMA conversion was confirmed by ^{19}F NMR. To ensure that all PFPMA moieties, even those below the NMR detection limit were removed, spermine (19.0 mg, 94.08 µmol for NP1 and 76 mg, 376.3 µmol for NP4) was added and the reaction was continued for 18 h at 50 °C. The DMSO/nanoparticle solution was dialyzed against water for one week with daily water exchange. After filtration with 0.45 µm PDVF filters, the aqueous solution was lyophilized yielding the nanohydrogel particles as orange powder.

The synthesis of NP2 and NP3 were conducted accordingly, using the indicated equivalents of crosslinker and spermine.[19]

3. Results and Discussion

As discussed above, a slightly crosslinked, but highly charged core with long polymer chains between the netpoints would be desirable to optimize the loading of the cationic core with siRNA.[20] A possible approach to achieve this, could be a decrease of the crosslinking density CLD. This could lead to a less rigid polymer network and larger pores so that more siRNA could enter the carrier system. Furthermore, the remaining reactive ester units could be used to functionalize the core further with secondary amines (positive charges) which additionally would improve the complexation capability. In this context, however, it had been observed that partially crosslinked systems were efficient in the uptake of siRNA but became larger and less stable than the densely crosslinked systems.[18] Thus, multifunctional crosslinkers with three or more primary amines get interesting. As they connect more than two polymer chains, they may lead to stable cationic nanohydrogel particles even with a reduced CLD. And this can help to obtain an improved therapeutic RNAi effect.

3.1. Synthesis of Four-Arm Crosslinker

As reference crosslinker, spermine as a two-arm oligoamine (see Figure 1A) was used. The new multifunctional (four-arm) crosslinker was synthesized in a six-step reaction containing four primary amines used for the crosslinking step and two secondary amines which facilitate the siRNA complexation. The synthesis plan is depicted in Figure 1B and the four-arm crosslinker was accessible by a peptide coupling of two protected lysine (**1.1**) derivatives with one protected spermine (**2.3**) molecule.

Lysine was selected as the carboxylic acid component for the coupling reaction, since by its additional amine group in the side chain, a multi-functional (four-arm) crosslinker would result after successful peptide coupling. The primary amines were protected using benzyl chloroformate (Cbz-Cl, Z) yielding *N,N*-(benzyloxy carbonyl)lysine (Z,Z-lysine, (**1**)) (see Figure S1, Supporting Information). Without protection of the amine groups, coupling between two lysine molecules would have led to various side reactions.

Spermine was used as the amine component for the peptide coupling. The secondary amines were selectively protected according to literature,[23] ensuring that the coupling reaction proceeds only by reaction of the primary amines (see Figure S2, Supporting Information). First, the protection of the primary amines using ethyl trifluoroacetate (TFA) was performed. The product was obtained as the TFA salt and was used without purification. Second, the remaining secondary amines were protected, again using the Cbz group. With these two different protection groups, an orthogonal deprotection of the primary amines was possible, affording the N4,N8-bis(benzyloxy carbonyl)spermine (N4,N8-Z,Z-spermine, (**2.3**)) (see Figure 1; Figure S2, Supporting Information).

Afterward, an EDC/HOBt mediated peptide coupling was performed. 2 equiv. of Z,Z-lysine were used as the carboxylic acid component and were reacted with N4,N8-Z,Z-spermine as the amine component. 1 equiv. of N4,N8-Z,Z-spermine

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was used to facilitate the amidation of both primary amines, yielding the protected precursor crosslinker (**3**) (see Figure 1). The successful coupling was confirmed by ^{1}H and DOSY NMR (see Figures S3 and S4, Supporting Information) since the corresponding signals are visible as one single diffusing species.

Finally, the Cbz protection groups of (**3**) were removed by hydrogenation, yielding the four-arm spermine-based crosslinker N1,N12-bis(2,6-di-amino-hexan-amido)spermine (**4**). HSQC NMR analyses presented in Figure 1C confirmed the successful synthesis of this new crosslinker. With this crosslinker at hand, nanohydrogel particles were prepared.

3.2. Synthesis of Cationic Nanohydrogels

Cationic nanohydrogel particles were synthesized according to the established procedure depicted in **Figure 2**, using the new multifunctional crosslinker (**4**) and/or spermine as reference. The amphiphilic block copolymer used for this procedure was synthesized via RAFT polymerization with a hydrophilic block consisting of the PEG-like monomer triethylene glycol monomethylether methacrylate (MEO_3MA) and a hydrophobic block made of the reactive ester pentafluorophenyl methacrylate (PFPMA). The block copolymer used here (see Figure 2A) consists of 24 hydrophilic units and 32 perfluorinated reactive ester units. Details of its characterization are presented in Supporting Information.

Such amphiphilic block copolymers can be pre-aggregated into micellar structures using a polar, aprotic solvent like dimethyl sulfoxide (DMSO) (i). The hydrophobic core of these aggregates can be crosslinked in a polymer analogous reaction of the PFPMA units with amine containing moieties (ii). During this crosslinking step the polarity of the core is switched from hydrophobic to cationic (hydrophilic), due to additional secondary amines incorporated by the crosslinker (see Figure 2B). The resulting cationic nanohydrogels are capable of complexing anionic oligonucleotide structures, like siRNA (iii), and thus, function as a suitable carrier system.

The used amphiphilic block copolymer $P(MEO_3MA)_{24}$-*b*-$P(PFPMA)_{32}$ (see Figure 2) was synthesized via RAFT polymerization as described previously (see Figure S5, Supporting Information).[19] According to the standard protocol for the nanohydrogel preparation, this amphiphilic block copolymer was dissolved in anhydrous DMSO and micellar self-assembly was supported by sonication.[9,10] These pre-aggregated structures were crosslinked using the new four-arm molecule 4 (and/or spermine as reference) as presented in Figure 2B. Thereby, we used stoichiometric amount of crosslinker (one primary amine per reactive ester unit), an excess of crosslinker (leading to incomplete reaction of the primary amines), or a reduced amount of crosslinker (leading to a reduced crosslinking density). Independent of these conditions, the primary amines of the crosslinker react quantitatively in a polymer analogous[16,17] reaction with the reactive ester moieties in the core and stable amide bonds are formed while the polymer chains in the core are connected.

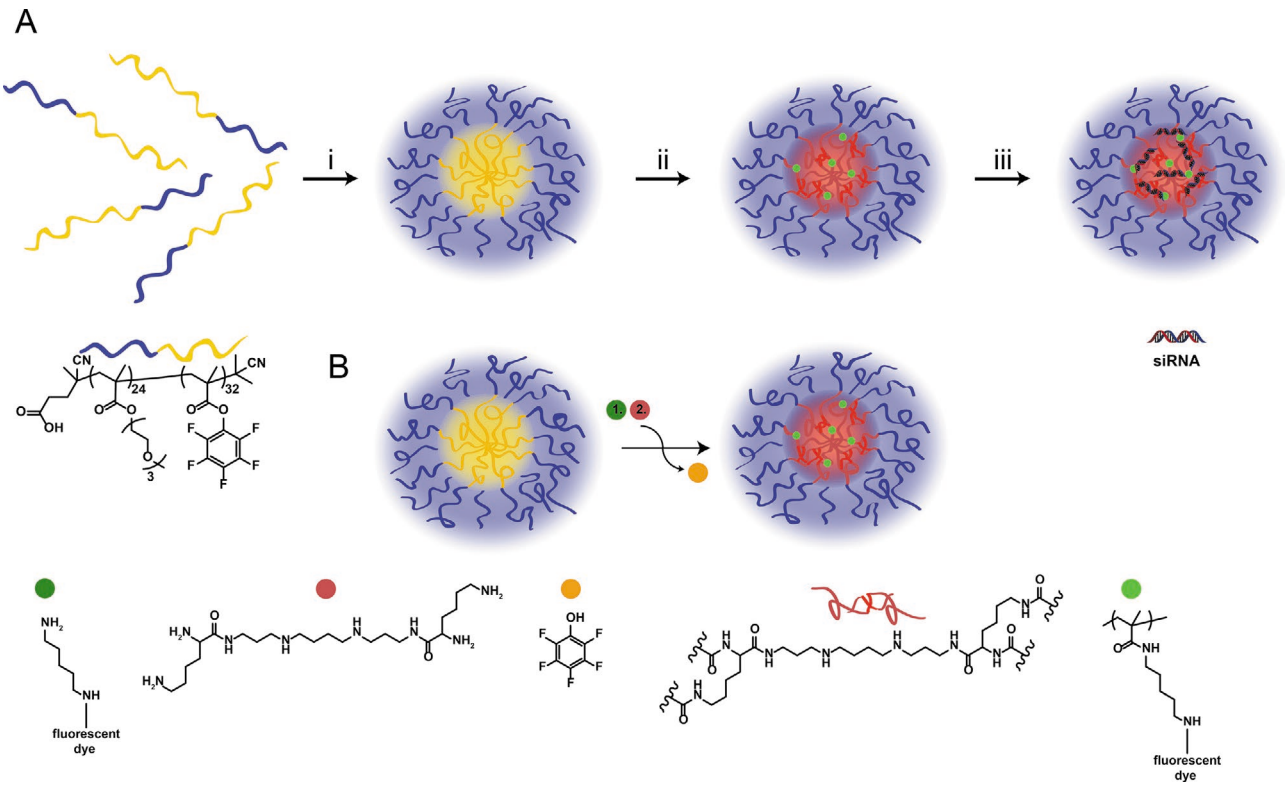

Figure 2. A) Concept of nanohydrogel particle synthesis. Amphiphilic block copolymers self-assemble into i) micellar structures which can be covalently crosslinked using ii) bi- or multifunctional amine containing molecules. The polarity of the core is switched during the crosslinking step, thus enable the complexation of iii) anionic siRNA; B) crosslinking step (ii) in detail.

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After the crosslinking step, an additional quenching step is performed to ensure complete conversion of all PFPMA units. In this context it should be noted that the conversion of the reactive ester units with primary amines proceeds quantitatively according to NMR-methods.[17] So this step is usually only performed to reach small traces of sterically hidden reactive ester units. For this purpose, an excess of spermine is added, which reacts mostly only with one primary amine unit, thus, facilitating the incorporation of additional primary amines as positively charged groups. The quenching step has only a minor influence on the crosslinking reactions for particle systems with a crosslinking density (CLD) of 100% since all reactive ester moieties should be converted already during the crosslinking. It gets, however, very significant for the nanohydrogels with a reduced amount of crosslinker, and thus, a reduced CLD, as NP4, as the remaining reactive ester moieties will be converted during the quenching step.

Different particles with varying crosslinking densities (CLD) and quenching equivalents were synthesized as summarized in **Table 1**.

According to the standard protocol, NP1 was prepared as the control system with 0.5 equiv. of spermine as crosslinker with two primary amines) yielding a CLD of 100%. Nanoparticle NP2-NP4 were prepared with the new four-arm crosslinker. For the preparation of NP2 0.25 equiv. of crosslinker (**4**) were used to obtain again a CLD of 100%. NP3 was synthesized with an excess of crosslinker, and thus, of primary amines by applying 0.5 equiv. of the four-arm crosslinker. Thereby, a maximal CLD of 100% was achieved while at the same time unreacted primary amines were incorporated in the core to increase the cationic charge. Finally, NP4 was synthesized to obtain a decreased CLD. For this purpose, 0.125 equiv. of the four-arm crosslinker was used resulting in a CLD of 50%. The remaining unreacted PFPMA units were then quenched with an excess of spermine (4 equiv.) to incorporate additional primary/secondary amines. The chemistry of the cationic structures after the different crosslinking processes are shown in **Figure 3**.

3.3. Nanoparticle Characterization and SiRNA Complexation

Dynamic light scattering (DLS) measurements of the nanoparticles revealed that the hydrodynamic diameter (D_h) of all cationic nanohydrogels (see Table 1) are in the same range. This is expected for properly crosslinked nanogels prepared from similar pre-aggregated polymeric micelles.[10,13,19] However, it is different for nanoparticles prepared with a deficit of bifunctional crosslinker (see Figures S7 and S8, Supporting Information).

At first, a hydrodynamic diameter of 26.6 nm for NP1 as the control particle (two-arm crosslinker) is obtained. The sizes of NP2 and NP3 (four-arm crosslinker) are only slightly higher (D_h = 32.4 and 33.6 nm, respectively). The particle NP4 with a hydrodynamic diameter of D_h = 26.5 nm was again very similar to the control particle NP1. This result is very important, since it demonstrates that the four-arm crosslinker allows it to reduce the crosslinking density without changing the size of the nanohydrogels (compare Supporting Information of ref.[18] and Figures S7 and S8, Supporting Information). According to earlier experiments, the size of the nanogels does not change after loading with siRNA.[9–11] Furthermore, all particles revealed narrow size distributions indicated by their mean PDI (see **Table 2** and Figure S6, Supporting Information).

Additional zeta potential (ζ) measurements confirmed the existence of cationic nanoparticles (ζ > +30 mV), and thus, the ability to complex anionic siRNA. They differed only slightly from particle to particle. While for NP1, NP2, and NP3 values of around 38–44 mV were obtained., the zeta potential of NP4 was slightly lower (+33 mV).

After characterization of these particles by DLS measurements, the evaluation of the complexation ratio by agarose gel electrophoresis was performed (see **Figure 4**). For this purpose, the intensity of free siRNA was determined at different N/P ratios.

The control particle NP1, which was crosslinked with spermine showed again a complexation ratio of 10:1 (weight-to-weight concentration w/w for NP:siRNA) as reported in earlier studies.[19] Spermine as crosslinker bears one secondary amine per primary amine and the complexation ratio of 10:1 corresponds to an N/P ratio of 9.3 normalized to the PFPMA units per polymer (see Table 2 and Supporting Information for calculations). For the particle system NP2 a decreased complexation capability should be obtained since the four-arm crosslinker bears only 0.5 secondary amines per primary amine. This assumption could be confirmed. Complete complexation of siRNA by NP2 was obtained at a ratio of 20:1 as shown in Figure 4. Thus, the number of secondary amines which are incorporated into the particle system directly effects the complexation ratio with siRNA. For NP3 a slight improvement for the siRNA complexation was achieved which goes in line with our assumptions. Using an excess of crosslinker leads to the incorporation of additional non-crosslinked primary amines

Table 1. Cationic nanohydrogel particles synthesized with four-arm crosslinker in comparison to two-arm crosslinker (spermine). Measurements were taken by dynamic light scattering (zeta-sizer). The data are given as the average of three measurements.

NP	CLD [%]	Equiv. crosslinker[a)]	Equiv. quencher spermine[a)]	D_h [nm]	Mean PDI	ζ [mV]
NP1[b)]	100	0.5	1	26.6 ± 0.4	0.19 ± 0.01	38.2 ± 0.6
NP2[c)]	100	0.25	1	32.4 ± 0.2	0.19 ± 0.01	40.7 ± 1.5
NP3[c)]	100	0.5	1	33.6 ± 0.7	0.24 ± 0.01	44.0 ± 0.7
NP4[c)]	50	0.125	4	26.5 ± 0.2	0.22 ± 0.01	33.1 ± 1.4

[a)]Equivalents regarding 1 equiv. of PFPMA per polymer chain; [b)]Using spermine as crosslinker; [c)]Using four-arm crosslinker for crosslinking.

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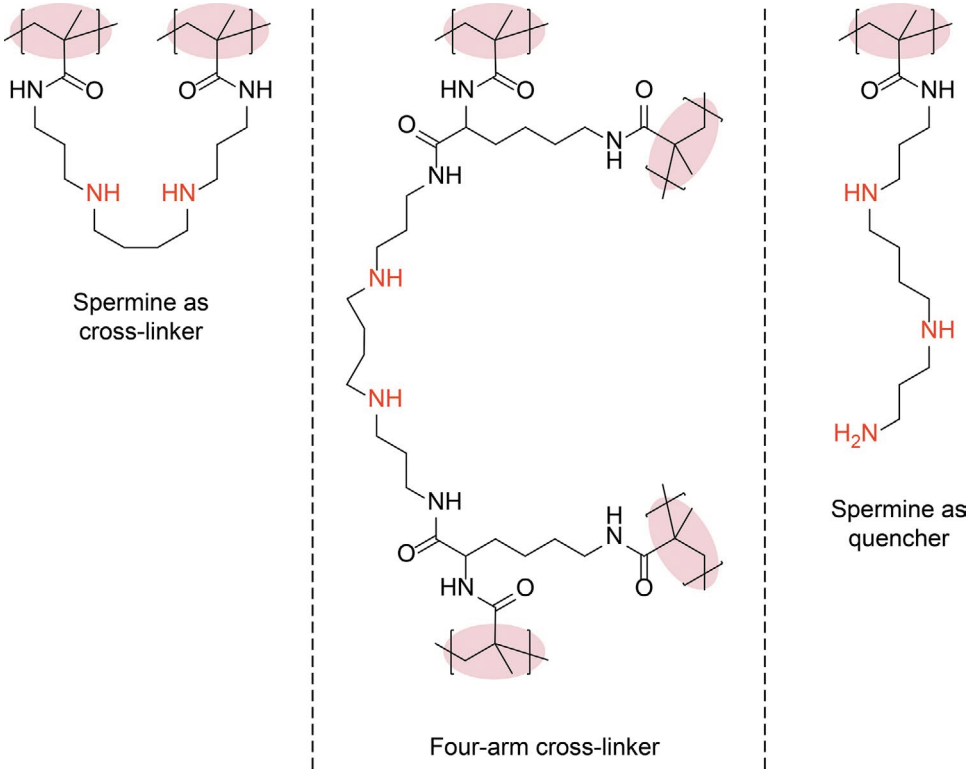

Figure 3. Chemical structures of different crosslinking and quenching molecules with their cationic amines) in red.

which then contribute to the improved siRNA complexation. Thus, for NP3 a complexation ratio of ≈10:1 was obtained (see Figure 4 (lower left) and Table 2).

For NP4, complete complexation was achieved at a ratio of nearly 5:1, determined by the proceeded disappearance of the siRNA signal compared to NP3 as shown in Figure 4 (lower right). NP4 was prepared with a decreased CLD of 50% and quenching of the remaining PFPMA units was achieved by an excess of spermine. Thus, the number of cationic groups is increased, while the crosslinking density gets decreased.

While the siRNA loading was, at first, determined on a simple weight to weight basis, it was afterward normalized to a N/P ratio with respect to the number of PFPMA units of the block copolymer, corresponding to the number of repeating units in the cationic core (N/P[c]). Both approaches led to equivalent results. Thus, the loading of the nanoparticles can be increased by a factor of 4 (indicated by the decrease of N/P) by varying the structure of the crosslinked hydrogels (NP2 to NP4), while keeping the new tetrafunctional crosslinker 4 constant. Thereby, NP2 in which crosslinker 4 acts as a tetragonal crosslinker, performs less than the reference NP1, while NP3 is about as effective as NP1. In NP3 the crosslinker 4 acts only like a bifunctional crosslinker (as in NP1) since an excess is used (see Table 1). Overall, NP4 is most effective in binding siRNA.

Table 2. Complexation ratios of cationic nanohydrogel particles. The ζ-potential is given as an average of three measurements.

NP	NP:siRNA[a)]	(+)[b)]	N/P[c)]	N/P[d)]	ζ [mV]
NP1	10:1	32	9.3	9.3	38.2 ± 0.6
NP2	20:1	16	17.4	8.9	40.7 ± 1.5
NP3	10:1	24	8.9	6.7	44.0 ± 0.7
NP4	5:1	56	4.0	7.0	33.1 ± 1.4

[a)]Weight-to-weight ratio determined by agarose gel electrophoresis; [b)]Cationic charges which are incorporated regarding the reactive ester block (X_n = 32), compare with Supporting Information; [c)]Normalized regarding the PFPMA units per polymer, see Supporting Information for the calculation of N/P ratio; [d)]Normalized regarding the cationic units per polymer, see Supporting Information for the calculation of N/P ratio.

Furthermore, the stability of these particles was confirmed with this agarose gel electrophoresis experiment. In a non-stable system, the siRNA would have been washed out of the carrier system during electrophoresis which would lead to a smear on the agarose gel.

To differentiate the impact of the varying positive charges and the role of the crosslinking density on the loading capacity, the N/P ratios were, later on, normalized with respect to the number of cationic amines, which were incorporated during the crosslinking step (N/P[d] in Table 2 and Figure 3). The calculation of the cationic charges per hydrophobic block is explained in Supporting Information and given in Table 2.

However, this new normalization of the N/P ratios regarding the cationic units per polymer demonstrated that the N/P[d] ratio varied only slightly (6.7 to 9.3) for all particle systems (see Table 2). Thus, the number of cationic charges is most important and the reduction of the crosslinking density as in NP2 (CLD 100%) to NP4 (CLD 50%) is of minor significance.

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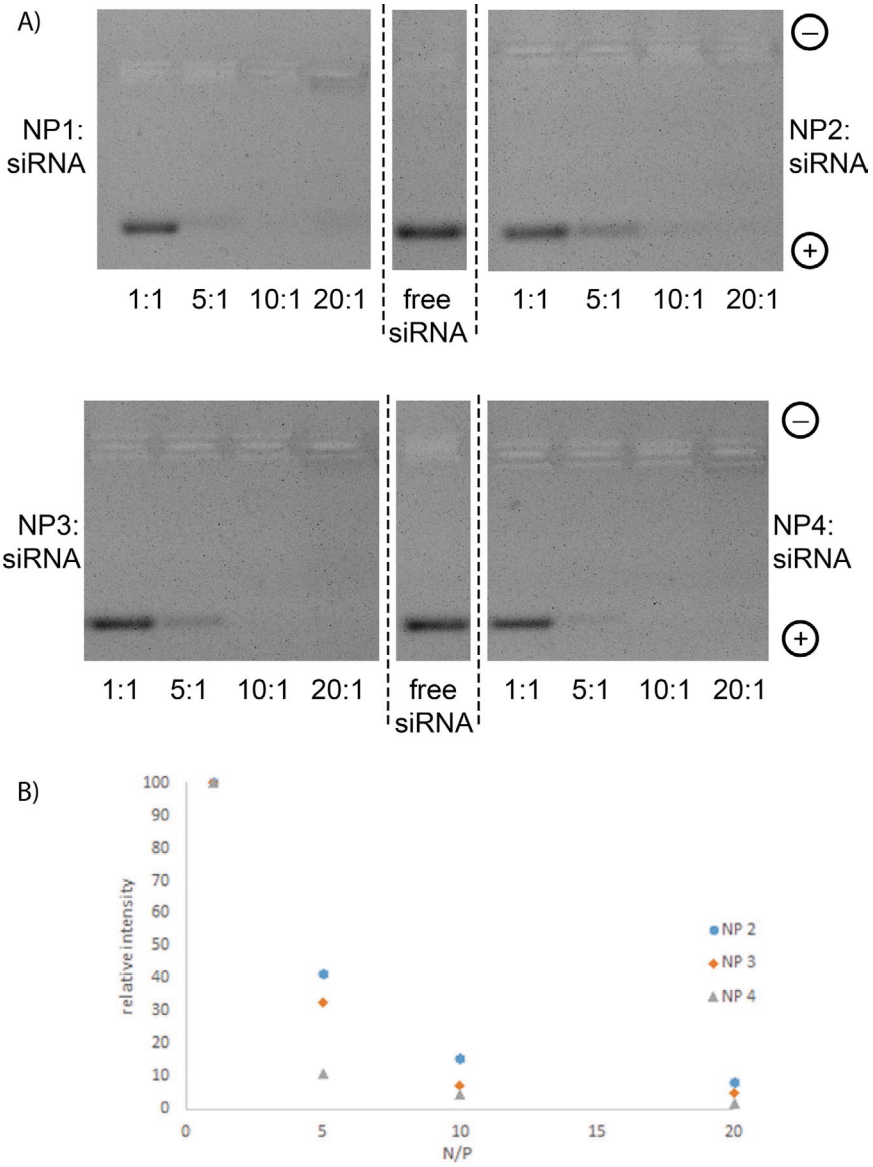

Figure 4. a) Determination of complexation ratio of siRNA loaded nanohydrogel particles (NP1–NP4) versus the N/P ratio by agarose gel electrophoresis. Original gels are shown in (a). b) From them the variation of the intensity of free siRNA with the variation of the N/P ratio was determined with the software ImageJ (see Figure S10, Supporting Information). The result for normalized "blackening curves" are shown in (b).

It leads only to a small reduction for the N/P from 8.9 (NP2) to 7.0 (NP4). In this context, it should be considered that overall also NP4 is rather densely crosslinked. Thus, also for NP4 the accessibility of the cationic charges by siRNA is limited.

4. Conclusion

In this study, we demonstrated that the complexation capability of cationic nanohydrogel particles with siRNA can be strongly varied and improved by working with a new tetrafunctional crosslinker.

At first, this tetrafunctional crosslinker opens the possibility to vary the amount of crosslinker per reactive unit of the polymer strongly without effecting the formation of stable cationic nanogels. This is, however, non-trivial as during the crosslinking reaction the thermodynamic driving force for micelle formation (very apolar, highly fluorinated core) is lost. Thus, only a quick and efficient crosslinking reaction can prevent the disintegration of the block copolymer micellar structure. Next, the possibility to perform the crosslinking also with an excess or a deficit of crosslinker, allows it to vary both the amount of cationic groups per cationic core and its crosslinking density. Both may affect the loading capacity for the anionic siRNA, whereby the quantitative evaluation of complexation revealed that the number of cationic charges is most important (at least for the crosslinking densities studied here). As a result, it becomes possible to increase the siRNA loading by a factor

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of 4 by varying the composition of the crosslinked core. Such an increased siRNA loading should lead to an improved therapeutic effect.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

cationic nanohydrogels, core crosslinked micelles, siRNA complexation, tetrafunctional crosslinkers

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