Synthesis of Alkyl-Modified Poly(sodium glutamate)s for Preparation of Polymer-Protein Nanoparticles in Combination with *N,N,N*-Trimethyl Chitosan

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ABSTRACT: The negatively charged, water-soluble, hydrophobically modified poly(sodium glutamate)s containing different amounts of alkyl grafts were synthesized. First, poly(γ-benzyl-L-glutamate) was prepared by ring-opening polymerization of the corresponding *N*-carboxyanhydride, which was in the next step aminolysed with octylamine. After removal of the remaining benzyl protective groups, the alkyl-modified poly(sodium glutamate)s [P(Glu-oa)] were obtained and, together with the oppositely charged *N,N,N*-trimethyl chitosan (TMC), used for the preparation of nanoparticles (NPs) of a recombinant granulocyte colony-stimulating factor (GCSF) protein by polyelectrolyte complexation method. It is observed that, beside electrostatic interaction, the hydrophobic grafts on poly(sodium glutamate)s significantly contribute to association efficiency (AE) with GCSF protein. The addition of TMC solution to the dispersion of GCSF/P(Glu-oa) complexes results in formation of much more defined NPs with high AE and final protein loading. © 2014 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2014**, *52*, 2976–2985

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INTRODUCTION Polymeric nanoparticles (NPs) have been widely investigated for drug delivery, including oral-delivery of pharmaceutically bioactive peptides and proteins.[1–6] A lot of attention has been given to NPs prepared from synthetic biodegradable and biocompatible polymers such as polycaprolactone,[7] polylactide,[8] and poly-D,L-lactide-*co*-glycolide copolymer.[9,10] Since these polymers are hydrophobic they are not ideal carriers for hydrophilic drugs like peptides and proteins. In such cases the NPs preparation procedures involve the use of organic solvents which can result in irreversible change of protein therapeutic activity unless special emulsion formulation procedures are employed.[11,12] In addition, a lack of functionality in these polymers makes their further modifications limited and, thus, an improvement of the interaction between the polymeric carrier and the protein/peptide drug is difficult. However, the NPs of bioactive macromolecules can be prepared under mild conditions by mixing them with an oppositely charged, water-soluble polymer (polyelectrolyte) in an aqueous medium to form the polyelectrolyte complexes particularly via noncovalent electrostatic interactions.[13–15] Various polymer combinations for protein NPs preparation have been reported with chitosan being one of the most commonly applied polymers, for example, chitosan/dextrane sulfate,[16,17] polyethyleneimine/dextran sulfate,[17,18] poly-L-lysine/dextrane sulfate,[17] chitosan/alginate,[19] chitosan/poly(γ-glutamic acid),[20,21] poly(vinyl pyrrolidone)/poly(acrylic acid),[22] etc. Chitosan is an ideal candidate for peroral drug-delivery due to its biocompatibility, biodegradability, muco-adhesion, and ability to transiently open the tight junctions between the intestinal epithelial cells, which results in facilitate transport of macromolecules through epithelia.[23] Recently, the quaternized chitosan derivatives were reported to have even better permeation-enhancing properties than chitosan itself. Additionally, NPs based on quaternized chitosan derivatives show greater stability due to enhanced electrostatic interaction, originating from the pH-independent positive charge, whereas the muco-adhesion of such NPs is preserved.[24–26]

Synthetic polypeptides are biodegradable and biocompatible polymers that can be used as an alternative to nondegradable synthetic polymers and as a substitute for some highly-functional natural polymers, which modification to prepare carriers with desired and reproducible properties, is difficult.[27] Synthetic homopolypeptides and copolypeptides of well-defined structure, narrow molecular-weight distribution

and various molecular architectures have been prepared under well-controlled experimental conditions by ring-opening polymerization of the *N*-carboxyanhydrides (NCA) of α-amino acids using primary amines as an initiator.[28] Since selection of α-amino acids, bearing various side groups, is very broad, the NCA monomers of different functionalities can be prepared. Together with the orthogonally protected amino acids' side groups and selective postpolymerization modification it is possible to prepare the polypeptides with versatile functionality. New approaches towards the design and synthesis of multifunctional polypeptides have been continuously developed.[29]

Synthetic polypeptides have been successfully applied in various drug delivery systems, mostly as amphiphilic hybrid copolymers with poly(ethylene glycol) (PEG).[30–32] Due to the amphiphilic nature of such copolymers, that is, PEG-*b*-poly(*β*-benzyl-L-aspartate),[33] PEG-*b*-poly(*N*-hexyl stearate-L-aspartamide),[34] PEG-*b*-poly(aspartic acid)-*b*-poly(D-leucine-*co*-tyrosine),[35] they form micelles in aqueous solutions into which low molecular-weight active pharmaceutical ingredients can be incorporated. Synthetic glycopolypeptides also show a great potential for drug delivery applications due to self-assembly behavior in solution and ability of carbohydrate for specific recognition of biological targets.[36] In addition, various combinations of aminoacids in the form of block or random copolymers have been synthesized, for example, poly(L-glutamic acid)-*co*-poly(L-lysine), which in dependence of their molecular structure self-assemble into pH-sensitive vesicles or micelles.[37,38] The most important parameters, defining the copolymer ability to form self-assembled structures are the copolymer chemical composition, architecture, and molecular weight of both blocks.[39]

The PEG-poly(amino acid) micelles have been applied for delivery of proteins as well. Heffernan and Murthy[40] prepared micelles with cross-linked core from a block copolymer of PEG and poly(L-lysine dithiopyridine) for delivery of negatively charged proteins. Harada et al.[41] prepared block copolymer of PEG and poly(glutamic acid) modified with octyl alcohol for intravenous delivery of a recombinant granulocyte colony-stimulating factor (GCSF) protein. The authors observed an immediate release of a part of protein, which was not incorporated into the micellar core but simply adsorbed on the outer PEG corona. The amount of initial protein release was smaller at lower initial protein loading, indicating limited final loading (FL) of these polymeric micelles.

In this work, we synthesized negatively charged, water-soluble, to a different degree hydrophobically modified poly(sodium glutamate)s (P(Glu-oa)) with randomly distributed octyl chains along the polypeptide backbone to study their ability for multiple type of interactions (electrostatic, hydrophobic interactions, and hydrogen bonds) with positively charged GCSF protein drug. Our goal was to prepare NPs with high association efficiency (AE) and high final protein loading per NP mass. Thus, we studied the influence of degree of octyl grafting on poly(sodium glutamate) backbone on the AE with the GCSF protein and subsequent NPs formation after addition of an oppositely charged *N*,*N*,*N*-trimethyl chitosan (TMC) to a suspension of GCSF/P(Glu-oa) complexes.

EXPERIMENTAL

Materials

Chemicals γ-benzyl-L-glutamate (BLG; 99%, Acros Organics), triphosgene (98%, Aldrich), hexylamine (99%, Sigma-Aldrich), octylamine (98%, Merck), 2-hydroxypyridine (2-HP; 97%, Aldrich), HBr (33 wt % in acetic acid, Acros Organics), HCl (37%, Merck), NaOH (p.a., Merck), chitosan ($M_w = 121$ kDa, 85% deacetylation, Sigma Aldrich), formaldehyde (37% water solution, Merck), MeI (99.5%, Sigma-Aldrich), formic acid (p.a., Kemika), NaCl (99%, Sigma-Aldrich), and solvents *N*,*N*-dimethylformamide (DMF, 99.8%, anhydrous Aldrich), *N*-methyl-2-pyrrolidon (NMP, 99,5%, Merck), ethanol (99,8%, Sigma-Aldrich), diethyl ether (99.7%, Merck), *n*-hexane (99%, Merck), trifluoroacetic acid (TFA, 99%, Aldrich), and tetrahydrofuran (THF, 99.9%, anhydrous, Sigma-Aldrich), were used as received. The GCSF protein was kindly provided by Lek Pharmaceuticals d.d.

Synthesis of Water-Soluble Alkyl-Modified Sodium Poly(glutamate)s

Synthesis of BLG N-Carboxyanhydride (NCA)

A BLG (5.00 g, 21.1 mmol) was suspended in dry THF (90 mL) and the suspension was heated up in an oil bath to 55 °C. A solution of triphosgene (3.38 g, 11.4 mmol) in dry THF (15 mL) was then added drop-wise. The reaction mixture was stirred for 75 min to obtain clear solution. Then, the reaction mixture was concentrated by vacuum evaporation and then hexane was slowly added. Afterwards, the reaction mixture was left to stand in the freezer overnight to ensure complete precipitation. The crude product was filtered, washed with hexane, and crystalized three more times from THF/hexane. (4.84 g, $Y = 87\%$).

Synthesis of Poly(γ-benzyl-L-glutamate) (PBLG)

The BLG NCA (4.00 g, 15.2 mmol) was dissolved in dry DMF (72 mL) and cooled down in an ice bath. A solution of hexylamine (0.335 mmol) in 4 mL of DMF was slowly added, and the reaction mixture was stirred for 24 h in an ice bath. Then, the reaction mixture was poured into an ice-cold water to precipitate the product. The precipitate was isolated by centrifugation (8000 rpm, 5 min), washed with water several times and freeze-dried (3.21 g, $Y = 96\%$).

Aminolysis of P(BLG)

Synthesis of P(BLG-oa) 4%. P(BLG; 1.00 g) and 2-HP (0.43 g, 4.57 mmol) were dissolved in dry DMF (15.2 mL). Then, octylamine (375 μL, 2.26 mmol) was added and the reaction mixture was stirred for 24 h at room temperature. Afterwards, the reaction mixture was poured into cold distilled water to precipitate the product. The precipitate was collected by filtration, washed with water, and dried in vacuum. Thus obtained [P(BLG-oa)] 4% product was washed

with hexane to remove the remaining octylamine (0.80 g, $Y = 73\%$).

Synthesis of P(BLG-oa) 13%. Similar procedure as above: P(BLG; 1.00 g), 2-HP (0.43 g, 4,57 mmol), octylamine (1.51 mL, 9.1 mmol), DMF (15.2 mL; 0.93 g, $Y = 85\%$).

Synthesis of P(BLG-oa) 24%. Similar procedure as above: P(BLG) (1.00 g), 2-HP (0.43 g, 4.57 mmol), octylamine (3.78 mL, 22.8 mmol), DMF (15.2 mL; 0.94 g, $Y = 86\%$).

Deprotection of P(BLG-oa) Samples

Synthesis of P(Glu-oa) 4%. P(BLG-oa) (0.81 g) was dissolved in TFA (20 mL). Then, HBr/acetic acid (33% w/w) was added (3.5 mL) and the reaction mixture stirred for 1 h. Afterwards, the reaction mixture was poured into diethyl ether to precipitate the product, which was collected by filtration. The product was neutralized with 0.1 M NaOH to pH = 8.5, to convert it into water-soluble alkyl-modified poly(sodium glutamate) [P(Glu-oa) 4%], which was then dialyzed (dialysis membrane with $MW_{cutoff} = 500$ Da) and finally freeze-dried (0.40 g, 70%).

Synthesis of P(Glu-oa) 13%. Similar procedure as above: P(BLG-oa) (0.85 g), TFA (21.2 mL), HBr/acetic acid (3.6 mL; 0.45 g, $Y = 71\%$).

Synthesis of P(Glu-oa) 24%. Similar procedure as above: P(BLG-oa) (0.91 g), TFA (22.8 mL), HBr/acetic acid (3.9 mL; 0.49 g, $Y = 69\%$).

Synthesis of TMC

Synthesis of N,N-Dimethyl Chitosan (DMC)

Chitosan (5.0 g) was suspended in formic acid (15 mL), followed by addition of 37% aqueous formaldehyde solution (20 mL) and distilled water (90 mL). The solution was heated to 70 °C and stirred for 118 h under reflux condenser. Then, the reaction mixture was partially evaporated to obtain viscous solution to which 1 M NaOH solution was added to set the pH of the medium to 12, at which the gel was formed. The gel was washed with water over a glass filter several times. DMC was dissolved in water at pH 4, that was adjusted with 1 M HCl. Solution was purified by dialysis for 3 days (dialysis membrane with $MW_{cutoff} = 12$ kDa). The remaining solution was freeze-dried to yield a white product (4.0 g, $Y = 70\%$).

Synthesis of TMC from DMC

DMC was dissolved in water (160 mL) and the pH was adjusted with 1 M NaOH to 11, at which the gel was formed. This step is performed to ensure deprotonation of DMC tertiary amino groups. Then, the gel was washed with water and acetone several times. Thus, prepared DMC (0.50 g) was suspended in NMP (140 mL) to which the iodomethane was added (4 mL). Suspension was heated to 40 °C and stirred. The precipitate (TMC) was isolated from ethanol/diethyl ether mixture (50/50) by filtration on a glass filter. After drying overnight, TMC was dissolved in an aqueous NaCl solution (10 wt %, 200 mL) and stirred for 18 h. Finally, TMC was purified by dialysis for 3 days (dialysis membrane with $MW_{cutoff} = 1$ kDa) and freeze dried to yield a white product (0.49 g, $Y = 80\%$).

Characterization

NMR Spectroscopy

The ^{1}H NMR spectra of samples were recorded, depending on their solubility, in DMSO-d_6 or D_2O on a 300-MHz Agilent Technologies DD2 spectrometer in the pulse Fourier Transform mode with both a relaxation delay and an acquisition time of 5 s. Tetramethylsilane (TMS, $\delta = 0$) and sodium 3-(trimethyl silyl) propionate-d_4 (TSP-d_4, $\delta = 0$) were used as the internal chemical-shift standards in DMSO-d_6 and D_2O, respectively.

Size-Exclusion Chromatography Coupled to a Multiangle Light-Scattering Photometer (SEC-MALS)

The SEC-MALS measurements were performed at room temperature using a Hewlett-Packard pump series 1100 coupled to a DAWN HELEOS laser photometer with a GaAs linearly polarized laser ($\lambda = 658$ nm) and to an Optilab rEX interferometric refractometer (RI) operating at the same wavelength as the photometer (both instruments are from Wyatt Technology Corp). The separation of chitosan and TMC was carried out on a Novema linear column with a pre-column (8 × 300 mm^2, Polymer Standards Service, molar mass range: up to 2 × 10^6 Da) in 0.2 M solution of sodium acetate/acetic acid in miliQ water at pH 4.4. The separation of PBLG sample was carried out on a PolarGel-L 8 μm column with a pre-column (300 mm length and 7.5 mm i.d., Polymer Laboratories, molar mass range: up to 30 kDa) in 0.05 M LiBr in *N*,*N*-dimethylacetamide at a flow rate of 1 mL/min. The masses of the samples injected onto the column were typically 1 × 10^{-4} g, whereas the solution concentrations were 1 × 10^{-3} g/mL.

The GCSF protein content in supernatants was determined by SEC coupled to a MALS-UV-RI multidetection system using a PROTEEMA column with a pre-column (100 Å, 8 × 300 mm^2, Polymer Standards Service, PSS, Germany, molar mass range: up to 1 × 10^5 Da) in 50 mM solution of $NaNO_3$ in miliQ water with 0.02% sodium azide. For the data acquisition and evaluation, the Astra 5.3.4 software (Wyatt Technology) was used. By knowing the protein specific UV extinction coefficient at 280 nm ($\varepsilon = 0.815$ mL/g cm) or its refractive index increment ($dn/dc = 0.186$ mL/g),[42] we calculated, from the concentration traces (UV or RI detector responses), the exact mass of the uncomplexed protein in the injected volume of the supernatant and, consequently, in the total volume of the supernatant/suspension (volume of all the solutions mixed for NP preparation). Based on these results the AE and the FL of GCSF in NPs were calculated according to eqs 1 and 2.

MALDI-TOF MS

MALDI-TOF mass spectra were recorded on a Bruker UltrafleXtreme MALDI-TOF-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The protected polypeptides were

recorded in a positive reflectron mode, while the deprotected polypeptides were recorded in a negative reflectron mode. The calibration was made externally with a Peptide calibration standard II (Bruker Daltonics) using nearest-neighbor positions. Matrix used was super DHB (mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) and sodium trifluoroacetate was used as a cationizer in the case of protected polypeptides.

Scanning Electron Microscopy (SEM)

SEM microscopy was used for morphological evaluation of NPs using a JSM-7001F Jeol (Japan) instrument with an acceleration voltage of 5.0 kV and a secondary electron detector. Freshly prepared NP suspensions were centrifuged at 15,000 rpm for 15 min, followed by resuspension of NPs in water to remove buffer salt. Such prepared suspensions were deposited on a double-sided carbon tape (diameter 12 mm, Oxford instruments, Oxon, UK), dried in a vacuum dryer at 25 °C for 5 h, and then the SEM images were taken.

Polyelectrolyte Complexation/NPs Formation and Their Characterization

The protein solution (4.4 mg/mL in 10 mM acetic buffer with 5% sorbitol, pH 4.5) was added drop-wise to the P(Glu-oa) polymer solution (2 or 3 mg/mL in 10 mM acetic buffer, pH 4.5) to form polyelectrolyte complexes. After stirring for a defined time, a solution of TMC (3 mg/mL) with opposite charge to the P(Glu-oa) polymer was added to GCSF/P(Glu-oa) complexes, which resulted in the formation of GCSF/P(Glu-oa)/TMC NPs.

The GCSF/P(Glu-oa) complexes and GCSF/P(Glu-oa)/TMC NPs were characterized by dynamic light-scattering (DLS) using a Zetasizer Nano ZS ZEN 3600 (4 mW He-Ne laser, 633 nm) from Malvern instruments, UK. Scattering light was detected at 173° by automatically adjusted laser attenuation filters and measurement position within the cell at temperature of 25 °C. For data analysis, the viscosity (0.8863 mPa) and refractive index (1.330 at 633 nm) of the distilled water at 25 °C were used. By DLS measurements we obtained the average hydrodynamic radius (D_h) of NPs and the size distribution that is described by the polydispersity index (PDI), which is a dimensionless number extrapolated from the autocorrelation function and ranges from the values close to zero for the uniform particles' size distribution and up to the values close to 1 for the broad size distribution of particles.

AE and FL of GCSF protein in complexes/NPs were determined indirectly after separating GCSF/P(Glu-oa) complexes or GCSF/P(Glu-oa)/TMC NPs from the dispersion media by centrifugation at 40,000 rpm for 20 min. Thus, obtained supernatants were analyzed for free protein content with SEC-MALS technique (see Experimental part).

The AE of GCSF protein with polymer(s) was calculated according to eq. 1. The difference between the total amount of GCSF protein used to prepare the complexes/NPs and the amount of free GCSF protein present in aqueous phase after centrifugation was divided with the total amount of GCSF protein added.

$$\text{AE\%} = \frac{\text{total amount of GCSF} - \text{unassociated GCSF}}{\text{total amount of GCSF}} \times 100 \quad (1)$$

The proportion of associated GCSF protein within complexes/NPs is defined as a FL by eq. 2. The FL represents a difference between the total amount of GCSF protein used for complexes/NPs preparation and the amount of non-associated GCSF protein, which was divided by the total amount of complexes/NPs.

$$\text{FL\%} = \frac{\text{total amount of GCSF} - \text{unassociated GCSF}}{\text{total amount of NPs}} \times 100 \quad (2)$$

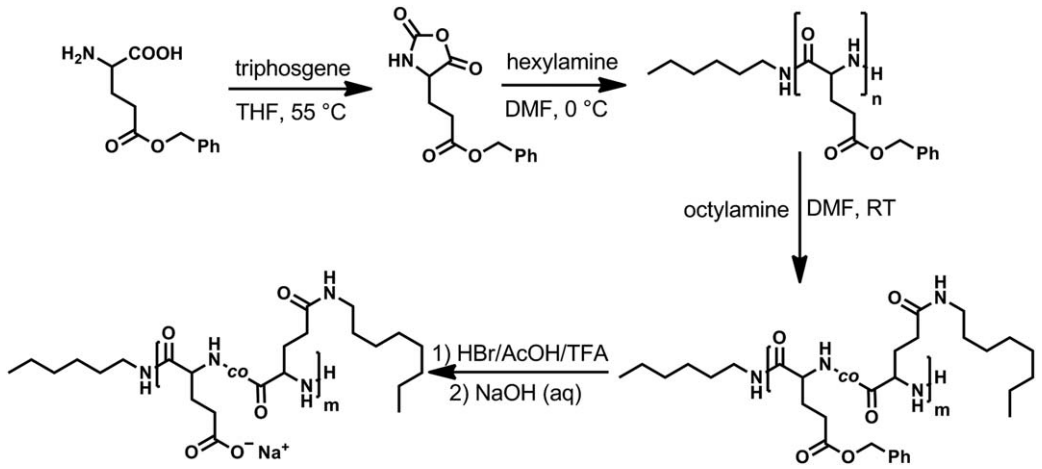
RESULTS AND DISCUSSION

Synthesis of Water-Soluble Alkyl-Modified Sodium Poly(glutamate)s

For preparation of hydrophobically modified poly(sodium glutamate)s [P(Glu-oa)] (Scheme 1) the BLG *N*-carboxyanhydride (NCA) was first prepared from the BLG and triphosgene. The BLG NCA was extensively purified by crystallization before it was polymerized since pure NCA is essential for good control over the polypeptide molar mass, the molar mass dispersity and the type of end groups.[28]

Polymerization of BLG NCA was initiated with primary hexylamine and performed at 0 °C to limit side-reactions, which are known to take place to a higher extent at room temperature.[43] The molar mass characteristics of thus prepared P(BLG) are: $M_n = 7.6$ kDa, $M_w = 8.0$ kDa, and $Đ = 1.05$ as determined by SEC-MALS [Fig. 1(A)]. Comparable molar mass with the peak apex at 8.2 kDa is obtained by MALDI-TOF MS. The mass spectrum shows a set of peaks with a difference of 219 Da between them, which corresponds to a benzyl glutamate repeating unit. The peak masses indicate macromolecules which are initiated with the hexylamine and terminated by the amine group as expected for a normal amine polymerization mechanism [Fig. 1(B)].[44,45]

Alkyl-modified P(BLG) samples with different content of alkyl grafts were synthesized by post-polymerization modification using partial aminolysis of P(BLG) with octylamine. Thus modified polypeptides consist of most probably randomly distributed alkyl chains along the backbone, which thus prevent supramolecular organization into well-defined micellar structures, typical for the block copolymers. Low content of alkyl side chains provides water-solubility of P(Glu-oa) copolymers. In addition, the formation of amide bond by aminolysis reaction avoids the use of carbodiimide coupling reagents, usually employed in postpolymerization modification of carboxyl groups, together with the associated side reactions (*N*-acylurea formation, etc.) and the issue with removal of urea as a side product.[46] The degree of P(BLG) aminolysis was controlled by changing the mole ratio of octylamine to benzyl glutamate repeat units. The polypeptide



SCHEME 1 Synthetic pathway for preparation of alkyl-modified poly(sodium glutamate)s, [P(Glu-oa)].

degradation during aminolysis was to a large extent prevented by the addition of 2-HP as a bifunctional catalyst.[47]

MALDI-TOF mass spectra of P(BLG-oa) samples reveal that all macromolecules contain the hexyl group at one chain end that originates from the initiator, whereas the amino end group presented in original P(BLG) samples transformed during aminolysis into a pyroglutamic group via intramolecular cyclization reaction with the adjacent benzyl ester group (Fig. 2). The intramolecular cyclization reaction to pyroglutamic end group is favored at elevated temperature,[43] whereas during aminolysis performed at room temperature, the cyclization is most likely additionally catalyzed by 2-hydroxypyrdine. A mass difference of 219 Da between the peaks of the main distribution corresponds to benzyl glutamate repeating unit, whereas a mass difference of 240 Da between the peaks of less intense peak distribution is due to octyl glutamide repeating unit. Thus, each peak of the main distribution shows sub-distributed peaks with the mass difference of 21 Da, which equals the difference between the benzyl ester and octyl glutamide units. Sub-distributed peaks indicate the polymer chains with the same degree of polymerization but different number of alkyl side chains. With increasing degree of alkylation the distributions of benzyl ester and octyl glutamide units become broader with accompanying signal overlapping, which make a detailed interpretation of MALDI-TOF mass spectra more difficult. Additionally, the mass spectra of P(BLG-oa) samples show very low intensity peak distribution with a mass difference of 129 Da, which reveals the presence of macromolecules with one deprotected carboxyl group. A peak distribution indicating macromolecules with two deprotected carboxyl groups was also observed, however these signals are of even lower intensity and strongly overlap with other signals. Thus, MALDI-TOF MS results reveal partial deprotection of benzyl protected carboxyl groups during aminolysis, which in our case is not a problem since the next synthetic step involves the removal of all benzyl protective groups. No degradation products originating from aminolysis and/or hydrolysis of the polypeptide backbone are observed in MALDI-TOF mass spectra of the P(BLG-oa) samples.

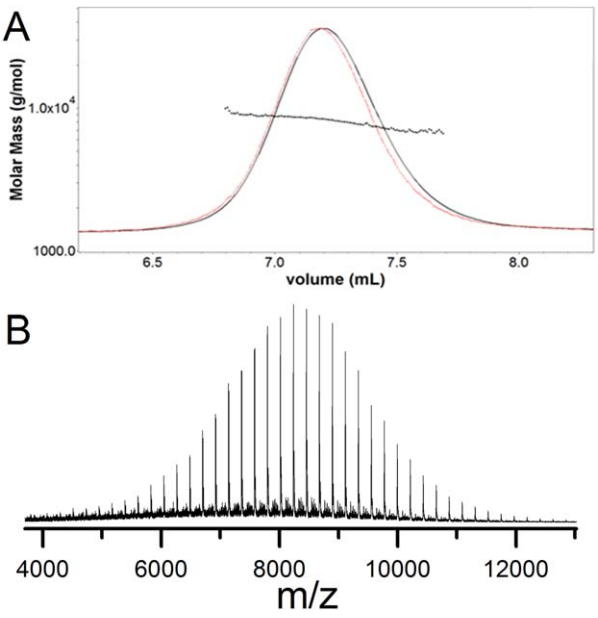


FIGURE 1 A: SEC-MALS chromatogram of P(BLG), black solid curve: refractive index detector response, red dotted line: light-scattering detector response at 90° angle, black squares: molar mass as a function of elution volume; (B) MALDI-TOF mass spectrum of P(BLG). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The benzyl protected carboxyl groups of partially alkylated P(BLG-oa) were deprotected under acidic conditions and, then, neutralized with NaOH to yield the water-soluble alkyl-modified poly(sodium glutamate)s, (P(Glu-oa)). The MALDI-TOF mass spectra of all three octyl modified and deprotected P(Glu-oa) polypeptides show peak apex at the expected molar masses. A slight increase in peak apex is noticed with increasing degree of P(Glu-oa) alkylation (Fig. 3). The sub-distribution of peaks due to the different chemical

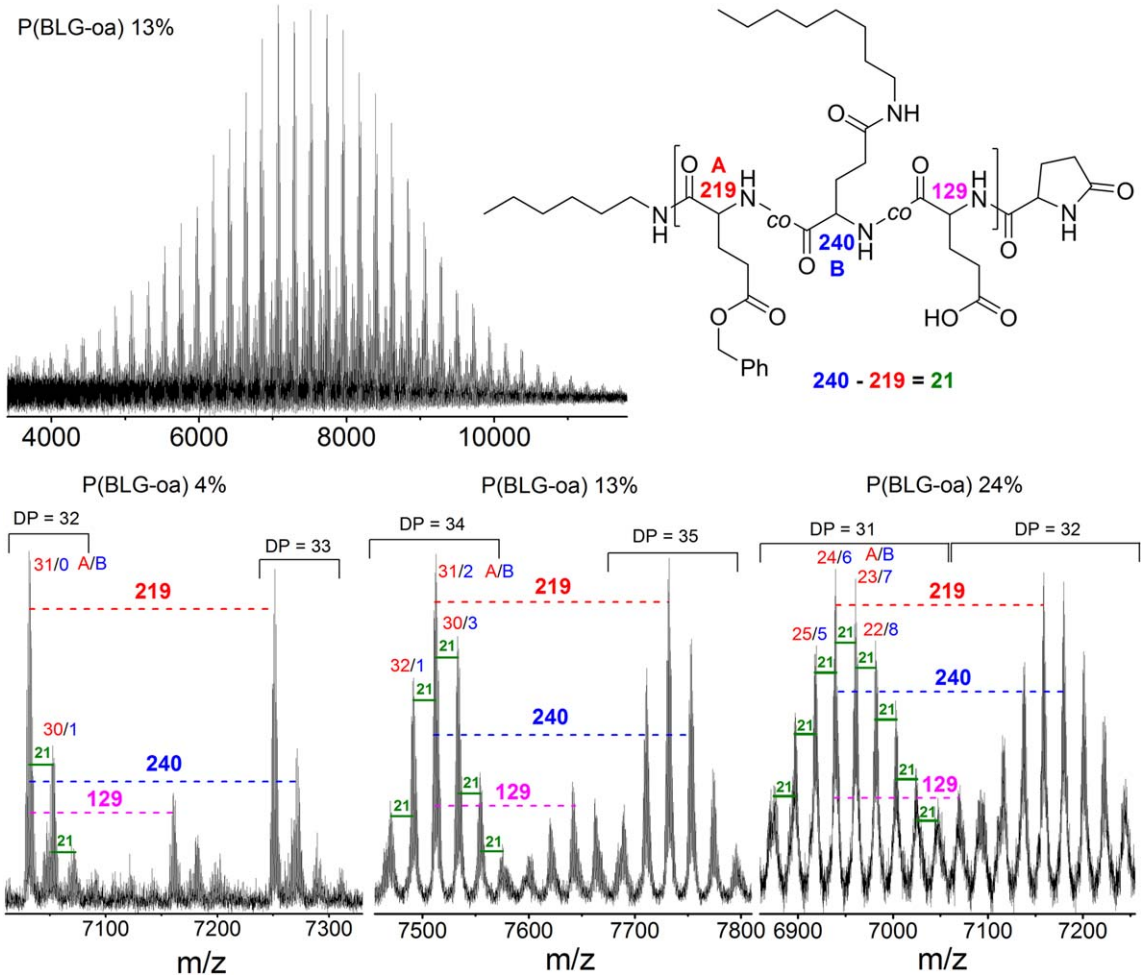


FIGURE 2 MALDI-TOF mass spectrum of P(BLG-oa) 13% (top) and magnified MALDI-TOF mass spectra of P(BLG-oa) samples with different degree of alkylation (bottom). The mass difference between the peaks, that is, 219, 240, and 129 Da belong to benzyl glutamate, octyl glutamide, and glutamic acid repeating units, respectively. The mass difference of 21 Da is due to different ratio between benzyl glutamate (A) and octyl glutamide (B) repeating units in the chains with the same degree of polymerization. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

composition of the chains with equal degree of polymerization is much more difficult to identify in P(Glu-oa) mass spectra since a difference between the octyl glutamide (240 Da) and the glutamic acid (129 Da) repeating units is 111 Da, which causes broadening of the peak distribution over several hundred Da and significant peak overlapping.

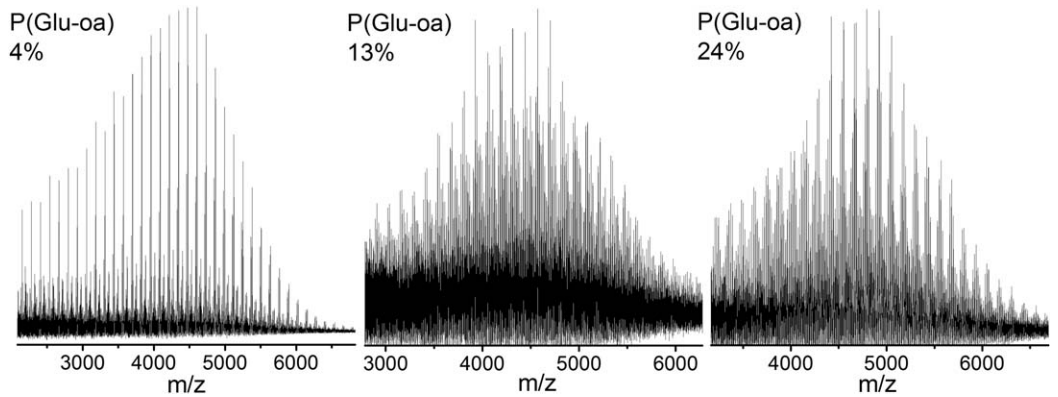


FIGURE 3 MALDI-TOF mass spectra of P(Glu-oa) samples after carboxyl group deprotection.

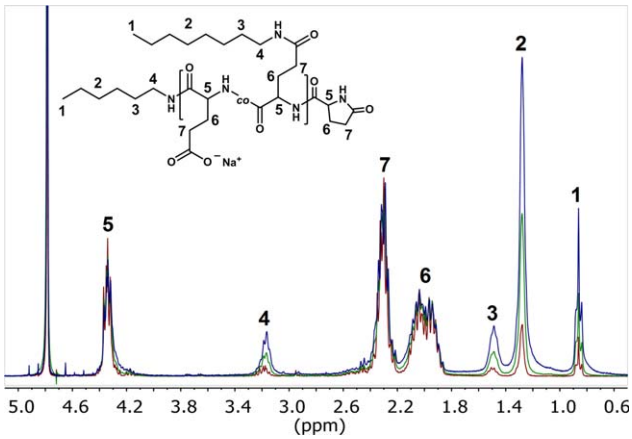
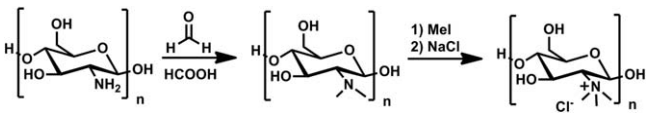


FIGURE 4 ^{1}H NMR spectra of alkyl-modified poly(sodium glutamate)s with different degree of randomly distributed alkyl chains: red spectrum: P(Glu-oa) 4%; green spectrum: P(Glu-oa) 13% and blue spectrum: P(Glu-oa) 24%. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



SCHEME 2 Synthetic pathway for preparation of TMC.

The degree of alkylation of P(Glu-oa) samples was calculated from the ^{1}H NMR spectra of products (Fig. 4) from the integral of the signals for the methyl group of the alkyl chain (signal 1) and the methyne group of the polymer backbone (signal 5) and is determined to be 4, 13 and 24% (% refers to alkyl chains per glutamate repeating units).

Synthesis of TMC

The TMC was prepared by quaternization of amine groups of DMC using MeI. The DMC was synthesized by Eschweiler-Clark reaction (Scheme 2).[48] Advantage of the described TMC synthetic procedure over direct chitosan quaternization using MeI and NaOH is in higher degree of quaternization and in absence of chitosan *O*-methylation as a side reaction, which allows further chitosan modification.[49]

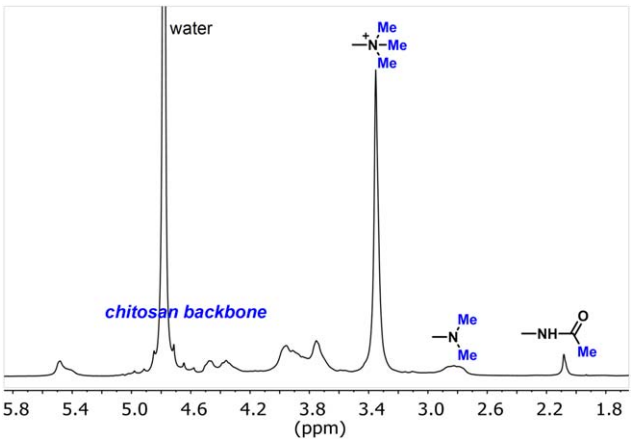


FIGURE 5 ^{1}H NMR spectrum of TMC with assignation of the main signals. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1 Experimental Conditions for Complex Preparation, AE, and FL of GCSF Protein in GCSF/P(Glu-oa) Complexes

	P(Glu-oa) V (1 mL) c (mg/mL)	GCSF c (4.4 mg/mL) V (mL)	Initial GCSF load (%)	AE (%)	FL (%)
P(Glu-oa) 4%	2	100	18.0	35	6
		200	30.6	37	11
	3	100	12.8	31	4
		200	22.7	41	9
P(Glu-oa) 13%	2	100	18.0	75	14
		200	30.6	84	26
	3	100	12.8	52	7
		200	22.7	67	15
P(Glu-oa) 24%	2	100	18.0	79	14
		200	30.6	88	27
	3	100	12.8	65	8
		200	22.7	79	18

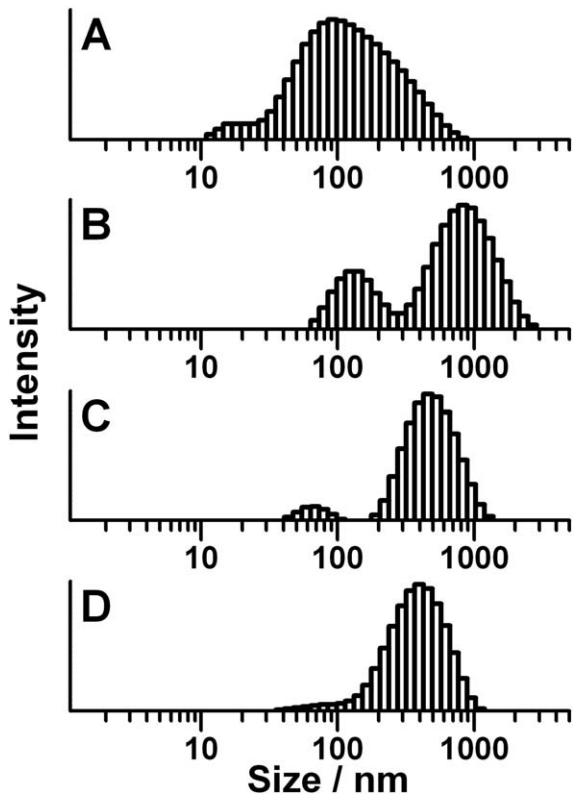


FIGURE 6 Histograms of 1 mL solutions of P(Glu-oa) 13% (3 mg/mL) with 100 μL of GCSF (4.4 mg/mL) without TMC (A) and with 10 μL (B), 30 μL (C), and 60 μL (D) of TMC (3 mg/mL).

TABLE 2 Experimental Conditions for NPs Preparation, AE, FL of GCSF Protein in GCSF/P(Glu-oa)/TMC NPs Together with the Size and Polydispersity of NPs

	P(Glu-oa) V (1 mL) c (mg/mL)	TMC[a] V (μL)	Initial GCSF load (%)	D_h (nm)	PDI	AE (%)	FL (%)
P(Glu-oa) 4%	2	10	17.8	267	0.198	45	8.9
		30	17.4	354	0.347	47	7.0
	3	10	12.7	282	0.243	54	7.3
		30	12.5	696	0.404	42	5.6
		60	12.2	1107	0.427	37	4.9
P(Glu-oa) 13%	2	10	17.8	325	0.486	70	13.2
		30	17.4	366	0.456	85	15.2
	3	10	12.7	332	0.648	69	9.1
		30	12.5	404	0.392	69	8.9
		60	12.2	409	0.400	73	9.2
P(Glu-oa) 24%	2	10	17.8	570	0.574	93	16.8
		30	17.4	474	0.523	93	16.4
	3	10	12.7	284	0.510	78	10.2
		30	12.5	422	0.471	76	9.8
		60	12.2	292	0.299	77	9.6

[a] Concentration of TMC was 3 mg/mL.

For all experiments 100 μL of 4.4 mg/mL GCSF protein solution was used.

The molar mass characteristics of TMC we have applied for NPs preparation are $M_n = 58$ kDa, $M_w = 114$ kDa, and $Đ = 1.9$ as determined by SEC-MALS. The ratio between the trimethylamine and dimethylamine groups is 5 to 1 as was calculated from the integral of the signals for the respective groups in ^{1}H NMR spectrum of TMC (Fig. 5).

NPs Formation from GCSF, P(Glu-oa) Polymers, and TMC

The ability of polymer to associate with protein is crucial for successful NPs preparation. In addition to efficient protein association, its FL in NPs should be high enough to obtain pharmaceutically acceptable system that is applicable for further dosage form formulation, which usually increases formulation overall mass.

The GCSF protein solution ($c = 4.4$ mg/mL, $V = 100$ or 200 μL) in a medium with pH 4.5, that is below the protein isoelectric point (pI 6.1), was added stepwise to a solution of P(Glu-oa) polymer ($c = 2$ or 3 mg/mL, $V = 1$ mL) with negative overall charge. After formation of the GCSF/P(Glu-oa) complexes, the oppositely charged TMC ($c = 3$ mg/mL, $V = 10$, 30 or 60 μL) was added to obtain well-defined NPs. The initial GCSF load is thus the amount of GCSF used divided by the total amount of all components.

The AE of GCSF protein with P(Glu-oa) polymers increases with the amount of alkyl grafts in poly(glutamate) in the following order: P(Glu-oa) 24% > P(Glu-oa) 13% >> P(Glu-oa) 4% (Table 1). A large increase in AE from the P(Glu-oa) 4% to the P(Glu-oa) 13% indicates significant contribution of hydrophobic interaction to complex formation. Higher solution concentration (3 instead of 2 mg/mL) of the P(Glu-oa) 4% polymer has negligible effect on AE of this particular polymer with GCSF, however, in the case of the P(Glu-oa) 13% and the P(Glu-oa) 24% the AE somewhat deteriorate, most probably due to the polymer self-association which results in lower probability of polymer to interact with the protein. However, higher amount of added GCSF solution (200 instead of 100 μL, $c = 4.4$ mg/mL) to P(Glu-oa) solution results in an improved AE between protein and polymer.

By subsequent addition of the positively charged TMC polymer solution to the dispersion of GCSF/P(Glu-oa) complexes the particles of larger size are formed (Fig. 6). The lowest amount

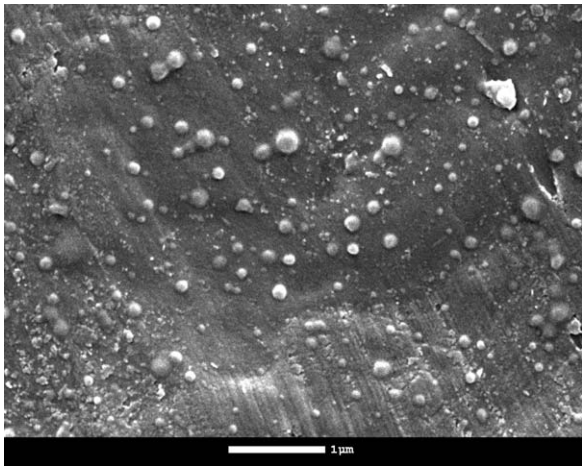


FIGURE 7 SEM of NPs prepared from 1 mL solution of P(Glu-oa) 13% (3 mg/mL) with 100 μL of GCSF (4.4 mg/mL) and 60 μL of TMC (3 mg/mL).

of added TMC results in bimodal size distribution [Fig. 6(B)]. However, by increasing the amount of TMC added, more defined particles are formed [Fig. 6(C,D)] without significant deterioration in AE (Table 2). The formation of NPs of protein, P(Glu-oa) and TMC was also confirmed by SEM (Fig. 7).

Higher amount of TMC can be added to the 3 mg/mL than to the 2 mg/mL P(Glu-oa) polymer solution before the precipitation of particles occurs, which reveals the ability of TMC to form stable NPs through electrostatic interaction and fine-tuning of final NPs size. Excellent AE as well as rather high final GCSF loading obtained with the P(Glu-oa) 13% and the P(Glu-oa) 24% polymers in combination with TMC demonstrate a potential for further investigation of these NPs for application in oral delivery of biopharmaceuticals.

CONCLUSIONS

Hydrophobically modified, negatively-charged, water-soluble poly(sodium glutamates) with different content of randomly distributed octyl chains in combination with TMC proved to be very effective in formation of GCSF protein loaded NPs using polyelectrolyte complexation method. The content of octyl grafts on poly(glutamate) backbone plays an important role in complexation efficiency of P(Glu-oa) polymers with GCSF protein. By optimization the ratio between the GCSF protein, P(Glu-oa, 13 or 24%) and TMC well-defined NPs with high AE and FL were prepared. Thus, a combination of P(Glu-oa) and TMC polymers for preparation of NPs loaded with GCSF provides minimal loss of protein drug during NPs preparation. This work demonstrates that polymers should be carefully designed to tune their properties in a way to efficiently interact with protein drug, which is a prerequisite for successful preparation of well-defined NPs with high FL.

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REFERENCES AND NOTES

1 C. Pinto Reis, R. J. Neufeld, A. J. Ribeiro, F. Veiga, *Nanomedicine* **2006**, *2*, 8–21.

2 F. Chiellini, A. M. Piras, C. Errico, E. Chiellini, *Nanomedicine* **2008**, *3*, 367–393.

3 R. Duncan, *Nat. Rev.* **2003**, *2*, 347–360.

4 D. Peer, J. M. Karp, S. Hong, O. C. Farokhzad, R. Margalit, R. Langer, *Nat. Nanotechnol.* **2007**, *2*, 751–760.

5 M. E. Davis, Z. Chen, D. M. Shin, *Nat. Rev.* **2008**, *7*, 771–782.

6 K. S. Soppimath, T. M. Aminabhavi, A. R. Kulkarni, W. E. Rudzinski, *J. Control. Release* **2001**, *70*, 1–20.

7 C. Damge, P. Maincent, N. Ubrich, *J. Control. Release* **2007**, *117*, 163–170.

8 H. Gao, Y. N. Wang, Y. G. Fan, J. B. Ma, *J. Control. Release* **2005**, *107*, 158–173.

9 Y. S. Nam, J. Y. Park, S.-H. Han, I.-S. Chang, *Biotechnol. Lett.* **2002**, *24*, 2093–2098.

10 A. Kumari, S. K. Yadav, S. C. Yadav, *Colloid Surf. B* **2010**, *75*, 1–18.

11 G. Liu, X. Hong, M. Jiang, W. Yuan, *Int. J. Nanomed.* **2012**, *7*, 4559–4569.

12 S. H. Choi, T. G. Park, *Int. J. Pharm.* **2006**, *311*, 223–228.

13 S. Boddohi, N. Moore, P. A. Johnson, M. J. Kipper, *Biomacromolecules* **2009**, *10*, 1402–1409.

14 S. Mizrahy, D. Peer, *Chem. Soc. Rev.* **2012**, *41*, 2623–2640.

15 H.-W. Sung, K. Sonaje, Z.-X. Liao, L.-W. Hsu, E.-Y, Chuang, *Acc. Chem. Res.* **2012**, *45*, 619–629.

16 Y. Chen, V. J. Mohanraj, J. E. Parkin, *Lett. Pept. Sci.* **2003**, *10*, 621–629.

17 M. Huang, S. N. Vitharana, L. J. Peek, T. Coop, C. Berkland, *Biomacromolecules* **2007**, *8*, 1607–1614.

18 W. Tiyaboonchai, J. Woiszwillo, R. C. Sims, C. R. Middaugh, *Int. J. Pharm.* **2003**, *255*, 139–151.

19 B. Sarmento, A. Ribeiro, F. Veiga, P. Sampaio, R. Neufeld, D. Ferreira, *Pharm. Res.* **2007**, *24*, 2198–2206.

20 Y.-H. Lin, F.-L. Mi, C.-T. Chen, W.-C. Chang, S.-F. Peng, H.-F. Liang, H.-W. Sung, *Biomacromolecules* **2007**, *8*, 146–152.

21 E.-Y. Chuang, K.-J. Lin, F.-Y. Su, F.-L. Mi, B. Maiti, C.-T. Chen, S.-P. Wey, T.-C. Yen, J.-H. Juang, H.-W. Sung, *J. Control. Release* **2013**, *172*, 513–522.

22 B. Deutel, M. Greindl, M. Thaurer, A. Bernkop-Schnürch, *Biomacromolecules* **2008**, *9*, 278–285.

23 K.A. Janes, P. Calvo, M. J. Alonso, *Adv. Drug Delivery Rev.* **2001**, *47*, 83–97.

24 A. Bayat, F. A. Dorkoosh, A. R. Dehpour, L. Moezi, B. Larijani, H. E. Junginger, M. Rafiee-Tehrani, *Int. J. Pharm.* **2008**, *356*, 259–266.

25 S. M. van der Merwe, J. C. Verhoef, J. H. M. Verheijden, A. F. Kotze, H. E. Junginger, *Eur. J. Pharm. Biopharm.* **2004**, *58*, 225–235.

26 F. Qian, F. Cui, J. Ding, C. Tang, C. Yin, *Biomacromolecules* **2006**, *7*, 2722–2727.

27 T. J. Deming, *Prog. Polym. Sci.* **2007**, *32*, 858–875.

28 N. Hadjichristidis, H. Iatrou, M. Pitsikalis, G. Sakellariou, *Chem. Rev.* **2009**, *109*, 5528–5578.

29 E. Garanger, S. Lecommandoux, *Angew. Chem. Int. Ed.* **2012**, *51*, 3060–3062.

30 Y. Bae, K. Kataoka, *Adv. Drug Delivery Rev.* **2009**, *61*, 768–784.

31 A. Lalatsa, A. G. Schätzlein, M. Mazza, T. B. Hang Le , I. F. Uchegbu, *J. Control. Release* **2012**, *161*, 523–536.

32 A. Lavasanifar, J. Samuel, G. S. Kwon, *Adv. Drug Delivery Rev.* **2002**, *54*, 169–190.

33 K. Kataoka, T. Matsumoto, M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto, G. S. Kwon, *J. Control. Release* **2000**, *64*, 143–153.

34 A. Lavasanifar, J. Samuel, G. S. Kwon, *J. Control. Release* **2002**, *79*, 165–172.

35 J. Rios-Doria, A. Carie, T. Costich, B. Burke, H. Skaff, R. Panicucci, K. Sill, *J. Drug Delivery*, in press, DOI: 10.1155/2012/951741.

36 C. Bonduelle, S. Lecommandoux, *Biomacromolecules* **2013**, *14*, 2973–2983.

37 J. Rodrıguez-Hernandez, S. Lecommandoux, *J. Am. Chem. Soc.* **2005**, *127*, 2026–2027.

38 Y. Huang, Z. Tang, X. Zhang, H. Yu, H. Sun, X. Pang, X. Chen, *Biomacromolecules* **2013**, *14*, 2023–2032.

39 I. F. Uchegbu, *Expert Opin. Drug Delivery* **2006**, *3*, 629–640.

40 M. Heffernan, N. Murthy, *Ann. Biomed. Eng.* **2009**, *37*, 1993–2002.

41 M. Harada, M. Ohuchi, T. Hayashi, Y. Kato, *J. Control. Release* **2011**, *156*, 101–108.

42 K. Rebolj, D. Pahovnik, E. Žagar, *Anal. Chem.* **2012**, *84*, 7374–7383.

43 G. J. M. Habraken, M. Peeters, C. H. J. T. Dietz, C. E. Koning, A. Heise, *Polym. Chem.* **2010**, *1*, 514–524.

44 E. Peggion, M. Terbjevich, A. Cosani, C. Colombini, *J. Am. Chem. Soc.* **1966**, *88*, 3630–3632.

45 M. Goodman, J. Hutchison, *J. Am. Chem. Soc.* **1966**, *88*, 3627–3630.

46 C. A. G. N. Montalbetti, V. Falque, *Tetrahedron* **2005**, *61*, 10827–10852.

47 A. De Marre, H. Soyez, E. Schacht, J. Pytela, *Polymer* **1994**, *35*, 2443–2446.

48 R. J. Verheul, M. Amidi, S. van der Wal, E. van Riet, W. Jiskoot, W. E. Hennink, *Biomaterials* **2008**, *29*, 3642–3649.

49 N. M. Alves, J. F. Mano, *Int. J. Biol. Macromol.* **2008**, *43*, 401–414.