Cellulose (2014) 21:3469–3485
DOI 10.1007/s10570-014-0362-8

ORIGINAL PAPER

Synthesis of chitosan-*graft*-poly(sodium-L-glutamate) for preparation of protein nanoparticles

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Received: 11 February 2014 / Accepted: 7 July 2014 / Published online: 15 July 2014

Abstract In this manuscript we have designed a synthetic approach for the preparation of a series of chitosan-*graft*-poly(L-glutamate) copolymers with different lengths of poly(L-glutamate) grafts. First, organosulfonic chitosan salt, soluble in DMSO, was prepared in order to effectively initiate ring-opening polymerization of γ-benzyl-L-glutamate *N*-carboxyanhydride. The chitosan-*graft*-poly(γ-benzyl-L-glutamate) copolymers were fully deprotected by applying tetrabutylammonium hydroxide. The molar mass characteristics and chemical composition of graft copolymers with various lengths of polypeptide grafts were determined by SEC-MALS, FT-IR and various NMR spectroscopic techniques. The synthesized chitosan-*graft*-poly(sodium-L-glutamate) copolymers were used in combination with trimethyl chitosan for the preparation of nanoparticles (NPs) of a recombinant granulocyte colony-stimulating factor (GCSF). The suspensions of NPs with typical average diameter of 200–300 nm were obtained with polydispersity index values below 0.26. The achieved loading efficiency was up to 95 % and the final loading of GCSF protein in NPs was up to 45 %. The time, temperature and pH stability of NPs was also studied.

Keywords γ-Benzyl-L-glutamate *N*-carboxyanhydride · Chitosan · Chitosan-*graft*-poly(γ-benzyl-L-glutamate) · Nanoparticles · Recombinant granulocyte colony-stimulating factor (GCSF) · Ring-opening polymerization

Abbreviations

1D	One-dimensional
2D	Two-dimensional
AcOH	Acetic acid
AE	Association efficiency
BGlu	γ-Benzyl-L-glutamate
Chi	Chitosan
Chi-*g*-PBGlu	Chitosan-*graft*-poly(γ-benzyl-L-glutamate)
Chi-*g*-PGlu	Chitosan-*graft*-poly(L-glutamate)
COSY	Correlation spectroscopy
CSA	(±)Camphor-10-sulfonic acid
DCA	Dichloroacetic acid
DLS	Dynamic light-scattering
DMAc	*N*,*N*-Dimethyl acetamide

Electronic supplementary material The online version of this article (doi:10.1007/s10570-014-0362-8) contains supplementary material, which is available to authorized users.

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DMF	*N*,*N*-Dimethyl formamide
DMSO	Dimethyl sulfoxide
d_H	Average hydrodynamic diameter
d*n*/d*c*	Refractive-index increment
$Đ_M$	Dispersity
FL	Final loading
FT-IR	Fourier transform infrared spectroscopy
GCSF	Granulocyte colony-stimulating factor
GE	Grafting efficiency
gHSQCad	Gradient Heteronuclear Single Quantum Coherence adiabatic version
GlcN	Glucosamine
Glu	Glutamate repeating unit
IL	Initial loading
MALS	Multi-angle laser light-scattering
M_n	Number-average molar mass
MSA	Methanesulfonic acid
M_w	Weight-average molar mass
NaAc	Sodium acetate
NCA	*N*-Carboxyanhydride
NMR	Nuclear magnetic resonance spectroscopy
NP	Nanoparticle
PBGlu	Poly(γ-benzyl-L-glutamate)
PdI	Polydispersity index
PEG	Poly(ethylene glycol)
PGlu	Poly(L-glutamate)
pI	Isoelectric point
RI	Refractive index
ROP	Ring-opening polymerization
SEC	Size-exclusion chromatography
TBAH	Tetrabutylammonium hydroxide
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TMC	Trimethyl chitosan
TMS	Tetramethylsilane
TMSI	Trimethylsilyl iodide
TMSPA	3-Trimethylsilyl-2,2′,3,3′-d_4-propanoic acid sodium salt

Introduction

Chitosan (Chi) is deacetylated chitin that is obtained from renewable resources. Due to its properties, e.g., mucoadhesivness, biocompatibility, biodegradability, gel forming ability, antimicrobial activity, low toxicity and non-immunogenicity, Chi is widely studied for application in biomedicine (Alves and Mano 2008; Carreira et al. 2010). Since Chi possesses a large number of functional groups (primary and secondary hydroxyl groups and primary amino group) it can be chemically modified in several ways (Ravi Kumar et al. 2004), e.g., carboxyalkylated, hydroxyalkylated, quaternized, hydrophobically modified, etc. For special applications, Chi or its oligomers were also glycosylated (Strand et al. 2008; Jain et al. 2012), dendronized (Aldana et al. 2010; Deng et al. 2011), and grafted (Qian et al. 2006; Bodnar et al. 2006; Yu et al. 2007; Carreira et al. 2010) to tune its properties for a particular application. Chitosan was thus grafted with poly(methyl methacrylate) to prepare polymeric carriers for oral drug delivery capable of encapsulating active pharmaceutical ingredients (Qian et al. 2006). Nanoparticles (NPs) prepared from these graft copolymers for example successfully protected insulin from harsh acidic and proteolytic conditions in the gastrointestinal tract and also sustained its release to the ileum (Qian et al. 2006).

Polypeptides or poly(amino acids) are biocompatible and biodegradable polymers that have found various applications in biomedicine (Kricheldorf 2006; Fante et al. 2011). Synthetic well-defined homo- and co-polypeptides with narrow molar-mass distribution are conveniently prepared by ring-opening polymerization (ROP) of the *N*-carboxyanhydrides of α-amino acids (NCA) (Deming 1997; Kricheldorf 2006; Hadjichristidis et al. 2009). As initiators for the ROP of NCA, the primary amines (Kricheldorf 2006), primary amine hydrochlorides (Dimitrov and Schlaad 2003), *N*-trimethylsilylamines (Lu and Cheng 2008) and organometallic compounds (Deming 1997) were applied (Cheng and Deming 2012). The NCAs are typically prepared by reaction of α-amino acids with phosgene or its analogues (Kricheldorf 2006).

Primary amine groups of Chi or their salts were successfully applied as initiators for ROP of various NCAs to synthesize the Chi-*graft*-poly(amino acid) copolymers (Chi et al. 2008; Kurita et al. 1988; Li et al. 2012; Liu et al. 2012; Nakamura et al. 2006; Xiang et al. 2009; Yu et al. 2007, 2011). Graft copolymerization of Chi took place under different conditions, namely in heterogeneous mixture of ethyl acetate and water at 0 °C (Kurita et al. 1988; Xiang et al. 2009), in

dimethyl sulfoxide (DMSO) in the presence of carboxylic acid additives (Nakamura et al. 2006) and by applying organosoluble 6-*O*-trityl Chi either in *N*,*N*-dimethyl formamide (DMF) (Yu et al. 2007, 2011) or in *N*,*N*-dimethylacetamide (DMAc; Li et al. 2012; Liu et al. 2012). A series of amphiphilic carboxymethyl-Chi-*graft*-poly(γ-benzyl-L-glutamate) was synthesized recently, where NCA ROP was conducted in DMSO (Huang and Jan 2014).

Deprotection of benzyl protected carboxyl group of poly(γ-benzyl-L-glutamate) (PBGlu) is known to be a very important synthetic step where much care should be taken due to the number of possible side reactions (Cheng and Deming 2012). The removal of benzyl ester group of PBGlu homopolymers or PBGlu block in block-copolymers has been performed under various conditions, i.e., by applying HBr/acetic acid (AcOH) in trifluoroacetic acid (TFA) (Babin et al. 2008; Han et al. 2013; Wang et al. 2011) or in dichloroacetic acid (DCA) (Wang et al. 2013; Zhuang et al. 2009), by methanesulfonic acid (MSA) in TFA (Nukui et al. 1991), by trifluoromethanesulfonic acid in TFA in the presence of thioanisole (Zhang et al. 2008), by aqueous NaOH (Han et al. 2013; Kukula et al. 2002; Liu et al. 2012; Rao et al. 2007; Tang et al. 2013), by trimethylsilyl iodide (TMSI) (Han et al. 2013; Subramanian et al. 2000) and also by catalytic hydrogenation (Deng et al. 2005). Various peptides' functional groups protected through ester bonds were hydrolyzed by applying Na_2CO_3 or other alkali carbonates in a methanol/water mixture (Kaestle et al. 1991). Another interesting approach to cleave the benzyl and alkyl ester protective groups from various (oligo)peptides has been achieved by using tetrabutyl ammonium hydroxide (TBAH) in different organic solvents as reported by Abdel-Magid et al. (1998).

The advantages of using NPs in oral protein-drug delivery include protection of the drug from degradation by enzymes and acidic pH as well as increased mucoadhesion and retention of NPs in the gastrointestinal tract (Gamboa and Leong 2013). Methods for NP preparation in general can involve the use of organic solvents, heating, sonication or vigorous agitation, which may be harmful to biopharmaceuticals (Plapied et al. 2011). On the other hand, the use of Chi based materials enables various mild NP preparation methods, e.g., covalent cross-linking, ionic cross-linking, polyelectrolyte complexation (Sarmento et al. 2007; Boddohi et al. 2009) and self-assembly (Mizrahy and Peer 2012). Polyelectrolyte complexes are formed spontaneously by direct electrostatic interaction of oppositely charged polyelectrolytes in solution (Boddohi et al. 2009; Mizrahy and Peer 2012; Sung et al. 2012; Giannotti et al. 2011). Chi based polyelectrolyte complexes were prepared and tested for application in oral insulin delivery, where poly(γ-glutamic acid) was applied as the negatively charged polyelectrolyte (Lin et al. 2007; Chuang et al. 2013). The NPs based on Chi and some of its derivatives, e.g., trimethyl chitosan (TMC), have been shown to be mucoadhesive and to reversibly open the tight junctions between the epithelial cells, thus promoting protein drug uptake in oral protein drug delivery (Lin et al. 2007; Mi et al. 2008; Chen et al. 2013). The benefit of TMC, relative to the application of unmodified Chi, for the preparation of NPs loaded with a protein drug lies in the permanent pH-independent positive charge and related enhanced solubility of TMC across a wide pH range (Mi et al. 2008).

Recombinant granulocyte colony-stimulating factor (GCSF), a hematopoietic growth factor that stimulates the differentiation, proliferation and functional activation of granulocytes is widely used to treat chemotherapy-induced neutropenia (Fox et al. 2009; Cox et al. 2014; Su et al. 2014). Since cancer patients, suffering from neutropenia, often receive more than ten injections of GCSF per chemotherapy round (Cox et al. 2014), the oral route of GCSF administration has been investigated in addition to the strategies that prolong a half-life of the injectable GCSF forms, i.e. PEGylation of GCSF (Molineux 2004), preparation of recombinant GCSF–immunoglobulin-1 fusion protein (Cox et al. 2014). The strategies for efficient oral delivery of GCSF biopharmaceutical have been investigated in the last two decades by applying, e.g. vitamin B12–GCSF conjugates (Habberfield et al. 1996), gastrointestinal mucoadhesive patch system (Eiamtrakarn et al. 2002), recombinant GCSF–transferrin fusion protein (Bai et al. 2005) and most recently by NPs formation (Su et al. 2014). GCSF is a 18.8 kDa glycoprotein with the pI value of 6.1. It contains 174 amino acid residues among which almost a half is hydrophobic (Arakawa et al. 1995; Ahmed et al. 2010). The crystal structure of GCSF reveals four long and one short helical elements (Young et al. 1997).

The aim of our work was to synthesize biodegradable and biocompatible copolymers based on the Chi

backbone and the poly(L-glutamate) (PGlu) grafts of different length. The chitosan-*graft*-poly(L-glutamate) (Chi-*g*-PGlu) copolymers together with trimethyl chitosan (TMC) were applied for the preparation of a novel carrier system in the form of NPs loaded with GCSF biopharmaceutical. NPs were prepared by spontaneous complexation of the oppositely charged polyelectrolytes under mild aqueous conditions. We studied the influence of Chi-*g*-PGlu chemical composition on association efficiency (AE) with GCSF and final GCSF loading in NPs. In addition, the time, temperature and pH stability of NPs was also evaluated.

Materials and methods

Materials

All solvents and reagents were used as received. Dry tetrahydrofuran (THF) (>99.9 %), dry DMSO (absolute, >99.5 %) and TBAH (40 wt% in H_2O) were obtained from Sigma-Aldrich, USA. THF (p.a.), *n*-hexane (p.a.), DMF (for GC), acetic acid (glacial, anhydrous), NaOH (>99 %) and $NaHCO_3$ (>99.0 %) were obtained from Merck, Germany. BGlu (>99 %) was obtained from Acros Organics, Belgium. Chitosan (Chitoscience, 95/5; 99 % deacetylated according to ^{1}H NMR) (Online Resource 1) was obtained from HMC, Germany. Triphosgene (>99.0 %) was obtained from Aldrich, USA. The (±)camphor-10-sulfonic acid (CSA) (>98 %) and NaCl (Ph Eur) were obtained from Fluka, Switzerland. TMC was prepared according to the published procedure (Verheul et al. 2008) (overall yield of two-step synthesis: 79 %, degree of trimethylation: 88 %, $M_n = 58$ kDa, $M_w = 114$ kDa, $Đ_M$: 1.9). The GCSF was kindly provided by Lek Pharmaceuticals d.d.

Synthesis

Synthesis of γ-benzyl-L-glutamate NCA (BGlu NCA)

BGlu (5.0 g, 21 mmol) was dispersed in dry THF (50 mL) under argon. A solution of triphosgene (3.1 g, 10 mmol) in dry THF (15 mL) under argon was added to the dispersion of BGlu. The reaction mixture was stirred at 55 °C for 90 min. Then the clear reaction mixture was concentrated under vacuum followed by precipitation in hexane. The product was crystallized three times from THF/hexane. Yield: 4.8 g (91 %).

Synthesis of chitosan (±)camphor-10-sulfonate (Chi CSA)

Chi (2.0 g, 12 mmol amino groups) was dispersed in deionized water (40 mL) and stirred for 30 min. CSA (2.9 g, 12 mmol) was added to the dispersion and stirred for an additional 4 h. The clear mixture was dialyzed (cut-off 1 kDa) against deionized water and freeze-dried. Yield: 4.7 g (96 %).

Synthesis of chitosan-graft-poly(γ-benzyl-L-glutamate) (Chi-g-PBGlu)

A detailed procedure for preparation of the Chi-*g*-$PBGlu_5$ graft copolymer is described, where the mole ratio $[NCA]_0$:$[NH_3^+]_0 = 5{:}1$ was applied. The same procedure was used to prepare the Chi-*g*-$PBGlu_{10}$ and Chi-*g*-$PBGlu_{15}$, where BGluNCA was added in 10 and 15 equiv., respectively, to the solutions of Chi CSA.

Chi CSA (1.309 g, 3.5 mmol, 1 equiv. ammonium groups) was dissolved in dry DMSO (150 mL) under argon. BGlu NCA (4.67 g, 17.7 mmol, 5 equiv.) was dissolved in dry DMSO (27 mL) under argon in a separate round-bottom flask and transferred to the solution of Chi CSA. A concentration of the starting $[NCA]_0$ solution was 0.1 M. The reaction mixture was stirred at room temperature for 3 days. The NCA conversion was monitored by ^{1}H NMR in DMSO-d_6 by disappearance of the signal for the BGlu NCA –NH– proton at 9.1 ppm. When the reaction was completed according to the NMR, the reaction mixture was poured into an ice-cold aqueous NaOH (5.3 mol, 250 mL) and vigorously stirred to precipitate the product. The crude product was washed with deionized water, dissolved in DMF and precipitated with dichloromethane to remove dioxopiperazine dimer of the BGlu NCA as a side product. Finally, the product was washed with water and then freeze-dried to obtain fine white powder. Yield: 3.5 g (78 %).

Synthesis of chitosan-graft-poly(sodium-L-glutamate) (Chi-g-PGlu)

Chi-*g*-$PBGlu_5$ (0.24 g, 0.96 mmol) was dissolved in DMF (9 mL) in an ice-bath. 40 % TBAH in water

(955 μL, 1.44 mmol) was slowly added during 2 min. After an additional 10 min, the reaction was quenched and the product precipitated by addition of 0.1 M AcOH. The suspension was centrifuged and the gel-like precipitate was dissolved in a minimal amount of saturated $NaHCO_3$, followed by dialysis (cut-off: 12 kDa) against 1 M NaCl and against deionized water. The product was freeze-dried to obtain the product Chi-*g*-$PGlu_5$. Yield: 53 mg, 70 %. The products Chi-*g*-$PGlu_{10}$ and Chi-*g*-$PGlu_{15}$ were prepared analogously.

Preparation of nanoparticles of GCSF

The Chi-*g*-PGlu copolymers were dissolved in water at a concentration of 2 mg/mL. The copolymer solution (1 mL, 2 mg/mL) was titrated with the solution of GCSF (4.4 mg/mL in 10 mM acetic buffer with 5 % sorbitol, pH 4.5). Then, the TMC solution (1 mg/mL) was added drop-wise. The resulting suspension was centrifuged (40,000 rpm, 20 min) and the supernatant was analyzed for the non-associated GCSF content to determine the AE and the final loading (FL) of GCSF in NPs.

Initial loading (IL), AE and FL were defined by the following equations:

$$IL\ (\%) = \frac{m(\mathrm{GCSF})_{total}}{m(\mathrm{Chi}-g-\mathrm{PGlu}) + m(\mathrm{GCSF})_{total} + m(\mathrm{TMC})} \times 100\ \% \quad (1)$$

$$AE\ (\%) = \frac{m(\mathrm{GCSF})_{total} - m(\mathrm{GCSF})_{supernatant}}{m(\mathrm{GCSF})_{total}} \times 100\,\% \quad (2)$$

$$FL\ (\%) = \frac{m(\mathrm{GCSF})_{total} - m(\mathrm{GCSF})_{supernatant}}{m(\mathrm{Chi}-g-\mathrm{PGlu}) + m(\mathrm{GCSF})_{total} - m(\mathrm{GCSF})_{supernatant} + m(\mathrm{TMC})} \times 100\,\% \quad (3)$$

The subscript "supernatant" refers to the amount of GCSF not associated with polymer into NPs. The amount of free GCSF was determined by SEC-MALS analysis of the supernatant. The IL is defined as the weight percent of the total added GCSF relative to the total mass of the applied Chi-*g*-PGlu, TMC (if added) and GCSF. The AE is defined as the percentage of the GCSF complexed in NPs. The difference between the total amount of GCSF used and the amount of GCSF that was detected in supernatant is attributed to the amount of GCSF within NPs. The FL is defined as the weight percent of the complexed GCSF relative to the total mass of the NPs.

To evaluate the time dependent stability of the GCSF loaded NPs, the suspensions of Chi-*g*-$PGlu_{15}$/GCSF (1/0.4, volume ratio) and Chi-*g*-$PGlu_{15}$/GCSF/TMC (1/0.4/0.05, volume ratio) were analyzed by DLS when freshly prepared and again after 16 days of incubation time at 25 °C.

Temperature stability of the GCSF loaded NPs was evaluated on Chi-*g*-$PGlu_{10}$/GCSF/TMC (1/0.4/0.05, volume ratio) and Chi-*g*-$PGlu_{15}$/GCSF/TMC (1/0.4/0.05, volume ratio) samples in a temperature range between 25 and 39 °C. A suspension was placed in the DLS cell and the temperature of the cell was raised by increments of 2 °C. Before DLS measurements at each temperature, the suspension was stabilized for 10 min. The effect of the pH of Chi-*g*-$PGlu_{10}$/GCSF/TMC (1/0.4/0.05, volume ratio) and Chi-*g*-$PGlu_{15}$/GCSF/TMC (1/0.4/0.05, volume ratio) suspensions on the size of NPs was evaluated by DLS measurements during titration of suspensions with 0.1 M NaOH solution.

Size-exclusion chromatography coupled to a multi-angle light-scattering detector (SEC-MALS)

SEC-MALS measurements were performed at room temperature using an Agilent Technologies pump series 1200 coupled to a multi-angle laser light-scattering detector Dawn Heleos (MALS) with a GaAs laser ($\lambda_0 = 658$ nm) and a refractive index detector Optilab rEX (RI), operating at the 658 nm wavelength (both instruments from Wyatt Technology Corp., USA). The separation of Chi sample was carried out

on a Novema linear column with a pre-column (8 mm × 300 mm, Polymer Standards Service, molar mass range: up to 2×10^6 Da) in 0.2 M solution of sodium acetate (NaAc)/acetic acid in miliQ water at pH 4.4. For Chi-*g*-PBGlu samples we used a PolarGel-M column with a pre-column (7.5 mm × 300 mm, Agilent Techn., molar mass range: up to 2×10^6 Da) and 0.1 M solution of lithium bromide (LiBr) in *N*,*N*-dimethylacetamide (DMAc) as the solvent and eluent. For Chi-*g*-PGlu samples the separations were carried out using a PolarGel-M column with a pre-column (7.5 mm × 300 mm, Agilent Techn., molar mass range: up to 2×10^6 Da) in 0.1 M solution of sodium nitrate ($NaNO_3$) in miliQ water at pH 10 with 0.02 % sodium azide. The GCSF content in supernatant was determined with a PROTEEMA GPC column with a pre-column (100 Å, 8 mm × 300 mm, Polymer Standards Service, PSS, Germany, molar mass range: from 1×10^2 to 1×10^5 Da) in 50 mM solution of $NaNO_3$ in miliQ water with 0.02 % sodium azide. The nominal eluents flow rates were 0.8 mL/min. The mass of the samples, except that of GCSF, injected onto the column was typically 1×10^{-4} g, whereas the solution concentration was 1×10^{-3} g/mL. The determination of absolute M_w and the calculation of M_n values from MALS detector require a sample-specific refractive-index increment (d*n*/d*c*), which was determined from the RI response assuming 100 % of sample mass recovery from the column. For the data acquisition and evaluation, Astra 5.3.4 software (Wyatt Technology Corp., USA) was utilized.

NMR spectroscopy

^{1}H, ^{13}C, ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H-^{13}C gradient Heteronuclear Single Quantum Coherence adiabatic version (gHSQCad) spectra were recorded in DMSO-d_6 or in D_2O on a Varian Unity Inova 300 instrument in the pulse Fourier-transform mode with a relaxation delay of 5 s and an acquisition time of 3 s. Tetramethylsilane (TMS, $\delta = 0$) and 3-trimethylsilyl-2,2′,3,3′-d_4-propanoic acid sodium salt (TMSPA, $\delta = 0$) were used as the internal chemical-shift standards in DMSO-d_6 and D_2O, respectively.

Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Spectrum One instrument (Perkin-Elmer, Inc., USA) upgraded with Universal ATR Accesory with diamond Top-plate-ZnSe. Typically, 16 scans were recorded at resolution of 4 cm^{-1} in the range of 650–4,000 cm^{-1}. Acquisition and processing of the data involved PE (Spectrum) Software.

Dynamic light scattering (DLS)

The particle size and polydispersity index (PdI) of NPs were measured 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 the automatically adjusted laser attenuation filters and the measurement position within the cell at 25 °C. For data analysis, the viscosity (0.8863 mPa) and the refractive index (1.330 at 633 nm) of the distilled water at 25 °C were used.

STEM microscopy

STEM micrographs were taken on a Zeiss Supra 35 VP at an acceleration voltage of 20.0 kV and working distance of 4.5–5 mm using a STEM electron detector. For STEM microscopy, water dispersion of the NPs was diluted with deionized water and a drop of dispersion was transferred to a Cu grid and dried.

Results and discussion

Chi-*g*-PBGlu synthesis

It has been reported that Chi can be dissolved in DMSO by salt formation (Sashiwa et al. 2000). Solubility test proved (*1R*)-(–)-10-camphorsulfonic acid to be a very promising organosulfuric acid for the preparation of a Chi sulfonate salt soluble in DMSO even when high-molecular weight Chi is used (Sashiwa et al. 2000). Chi CSA was thus prepared under mild conditions in a single step by dissolving Chi and CSA in water (Fig. 1), followed by purification by dialysis and subsequent freeze-drying to obtain white fluffy solid. The ROP of NCA was already successfully initiated by applying primary amine hydrochloride macroinitiator to prepare linear block copolymers (Dimitrov and Schlaad 2003) and by primary amine carboxylate macroinitiator to prepare graft copolymers (Nakamura et al. 2006). Therefore, we used a

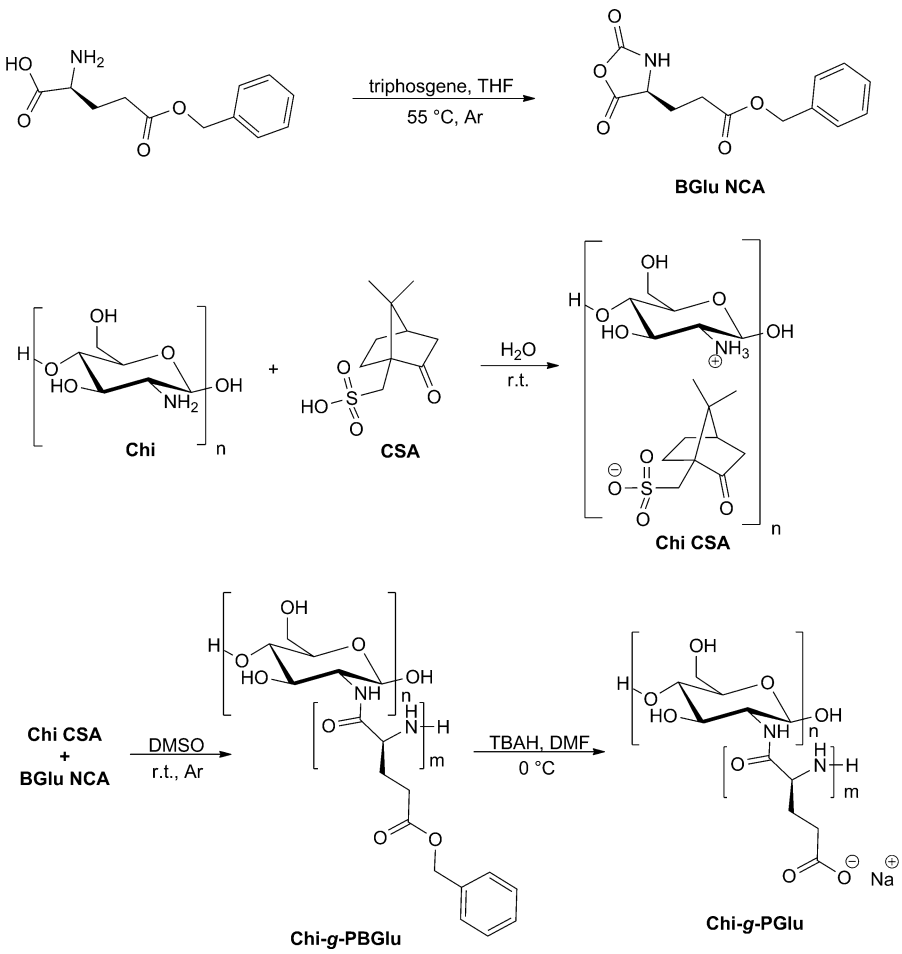

Fig. 1 Reaction pathway for preparation of Chi-*g*-PGlu copolymers

primary amine sulfonate salt, Chi CSA, as a macro-initiator for the preparation of Chi-*g*-PBGlu with various lengths of the grafted PBGlu chains (Fig. 1). As compared to the syntheses involving 6-*O*-trityl Chi (Nakamura et al. 2006; Yu et al. 2007; Li et al. 2012; Liu et al. 2012), it is important to note that in our case after the graft copolymerization completion there was no need to deprotect the hydroxyl groups of the Chi backbone, since they had not been protected or modified in the first place.

The structure of Chi CSA was characterized by ^{1}H–^{1}H COSY and ^{1}H–^{13}C gHSQCad 2D NMR experiments in addition to ^{1}H and ^{13}C experiments (Online Resource 2-5). Due to peak overlapping, some peaks of the Chi moiety protons (H-1, H-2 and H-3) were assigned by COSY (Online Resource 4) and the assignation was further confirmed by gHSQCad experiment (Online Resource 5). Thus, in the ^{1}H NMR spectrum of Chi CSA, the Chi moiety shows the signals at 4.81 ppm for H-1, at 2.86 ppm for H-2, at 3.50 ppm for H-5. Other Chi protons that are bound to the carbons are assigned to a broad peak at 3.71 ppm (Online Resource 2). To assign the signals of the CSA moiety, gHSQCad spectrum (Online Resource 5) was very helpful, since COSY spectrum (Online Resource 4) is rather complex in the region between 1.0 and 3.0 ppm and, as observed in gHSQCad, the signals of CSA protons in positions c and d overlap at 1.30 ppm.

In the next step, Chi CSA was dissolved in dry DMSO under argon. Then the solution of BGlu NCA (5, 10 and 15 equivalents to individual ammonium group of Chi CSA) in dry DMSO was added via syringe to the solution of Chi CSA. NCA ROP was conducted at room temperature. Complete conversion of the BGlu NCA was achieved in 3 days as confirmed by monitoring the disappearance of the N–H signal at 9.1 ppm, characteristic of BGlu NCA, by ^{1}H NMR spectroscopy in DMSO-d_6 (Online Resource 6). The

ROP of NCA proceeded without the need of heating the reaction mixture as it was proposed by Dimitrov and Schlaad (2003) for NCA ROP initiated by primary amine hydrochloride based macroinitiators in DMF to prepare linear block copolymers. After reaction completion, the product was precipitated by pouring the reaction mixture into an ice cold aqueous NaOH solution (1.5 equivalents relative to CSA). By NaOH addition, the ammonium groups were deprotonated and the CSA moiety was effectively removed from the resulting reaction products as confirmed by absence of the CSA protons in ^{1}H NMR spectra of grafted products (Fig. 2a, Online Resource 2, Online Resource 7).White precipitate was collected by centrifuge and washed with water. The resulting crude Chi-*g*-PBGlu graft copolymers were further purified by dissolving in DMF and precipitation with dichloromethane in order to remove dioxopiperazine dimer (Brulc et al. 2011) of the BGlu NCA as a side product. Thus purified Chi-*g*-PBGlu copolymers were freeze-dried. The yields of the grafting reactions were above 70 % (Table 1).

As observed in the COSY spectrum of the Chi-*g*-PBGlu$_5$ (Online Resource 8), the proton signals at δ (ppm) 4.30, 4.04 and 3.17 are all correlated to the BGlu side chain signals. Upon acidification with TFA, the peak at 3.17 ppm shifted downfield to 3.87 ppm (spectrum not shown), indicating that it belongs to the α-proton of the terminal grafted BGlu residue. In gHSQCad spectrum of Chi-*g*-PBGlu$_5$, the chitosan backbone signals had very low intensities and thus only H-6 (3.59 ppm) and H-5 (3.23 ppm) protons were assigned by gHSQCad spectrum (Online Resource 9).

Molar mass characteristics of the Chi and Chi-*g*-PBGlu were assessed by SEC-MALS measurements. For the supplied Chi, the number and weight average molar masses of 1.9×10^4 and 3.4×10^4 g/mol, respectively, and molar mass dispersity of 1.8 were

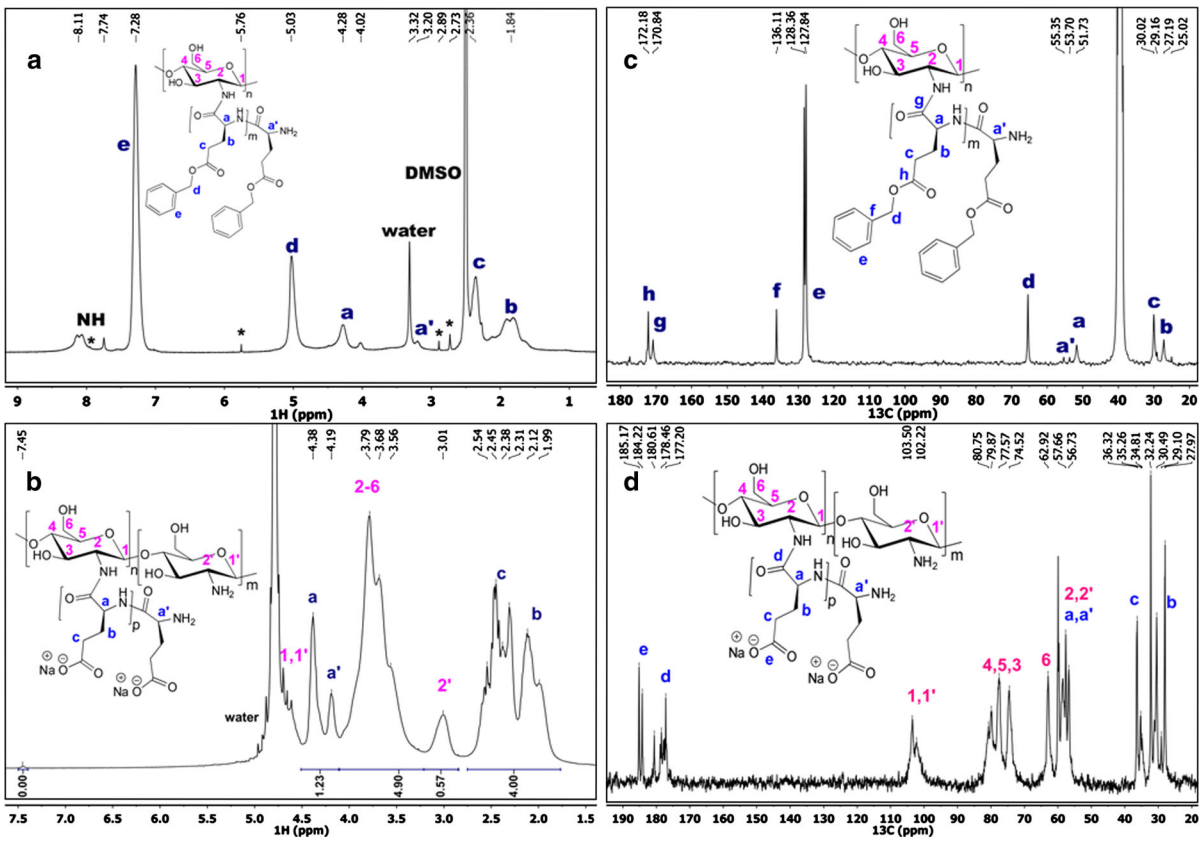

Fig. 2 ^{1}H NMR spectra of **a** Chi-*g*-PBGlu$_5$ in DMSO-d_6 and **b** Chi-*g*-PGlu$_5$ in D_2O. ^{13}C NMR spectra of **c** Chi-*g*-PBGlu$_5$ in DMSO-d_6 and **d** Chi-*g*-PGlu$_5$ in D_2O

Table 1 Results of SEC-MALS measurements for Chi and Chi-*g*-PBGlu samples, conversion of BGlu NCA and reaction yields

	Estimated values[a]	SEC-MALS			Conversion of BGlu NCA[b] (%)	Reaction yield (%)
	M_n (10^5 Da)	M_n (10^5 Da)	M_w (10^5 Da)	$Đ_M$		
Chi	–	0.19	0.34	1.8	–	–
Chi-*g*-PBGlu$_5$	1.46	1.25	2.82	2.3	>99	78
Chi-*g*-PBGlu$_{10}$	2.73	2.18	4.48	2.1	>99	73
Chi-*g*-PBGlu$_{15}$	4.00	3.35	6.95	2.1	>99	71

[a] M_n values were calculated based on M_n(Chi) = 1.9 × 10^4 Da, degree of Chi deacetylation 99 % and theoretical degrees of polymerization of the grafted PBGlu being 5, 10 and 15, respectively

[b] BGlu NCA conversion monitored by ^{1}H NMR spectroscopy

obtained. The molar mass averages of Chi-*g*-PBGlu reaction products were significantly higher (order of magnitude 10^5) than the ones of the starting Chi, indicating successful grafting of the PBGlu on the Chi backbone (Table 1).

Chi-*g*-PGlu synthesis

In an attempt to remove the benzyl ester group from Chi-*g*-PBGlu copolymers, catalytic hydrogenation applying palladium on charcoal catalyst was used, however, the starting Chi-*g*-PBGlu materials returned intact. The deprotection procedures applying HBr, MSA or TMSI resulted in severely degraded products with M_n below 10 kDa (data not shown). The deprotection reactions applying either NaOH or Na_2CO_3 resulted in degraded products with bimodal molar mass distribution ($Đ_M$ above 3, data not shown).

Since the cleavage of the benzyl and alkyl ester protective groups of various peptides is effective by using TBAH in various organic solvents as reported by (Abdel-Magid et al. 1998), this procedure was applied in DMF solution at 0 °C to remove the benzyl protecting group of Chi-*g*-PBGlu. Remarkably, it took only 10 min for almost complete benzyl ester removal (>99 %). Furthermore, the reaction under these conditions proceeded homogeneously. TBAH is readily quenched by acidification of the reaction mixture with aqueous acetic acid. By acidification, the product precipitated and is then conveniently isolated by centrifugation. To completely exchange tetrabutylammonium cations from the resulting precipitate with the sodium cations, the product was dialyzed against 1 M NaCl.

In ^{1}H NMR spectrum of Chi-*g*-PGlu$_5$ sample (Fig. 2b) a broad signal between 1.75 and 2.75 ppm was assigned to the Glu side chain protons. The proton signal at 3.01 ppm was assigned to the 2′ proton of the not substituted Chi moieties and the assignation was further confirmed by gHSQCad (Fig. 3) and COSY (Online resource 10) spectra.

Chemical composition, i.e. the ratio between the Glu and the Chi repeating units (Glu:NH_2) was estimated by dividing the normalized integral value of the Glu signals b and c (four protons) with the normalized integral value of the Chi protons 2–6 and 2′ (six protons) (Fig. 2b, Online resource 11) as defined in Eq. 4:

$$Glu{:}NH_2 = \frac{(integral\, b\&c)/4}{(integral\, 2-6\&2')/6} \quad (4)$$

The obtained results showed an increase in Glu:NH_2 ratio from 1.2 for the Chi-*g*-PGlu$_5$ to 2.2 for the Chi-*g*-PGlu$_{15}$ (Table 2).

Grafting efficiency (GE) was calculated as the proportion of the grafted GlcN units of the Chi backbone relative to the sum of the grafted and the non-grafted GlcN units. Since the 2′ signal of the non-grafted GlcN units does not overlap with any other signal, the proportion of the non-grafted units (defined as 1 − GE) can be determined from the ^{1}H NMR spectra of the Chi-*g*-PGlu copolymers by the following equation:

$$1 - GE = \frac{6 \times integral\, 2'}{integral\, (2-6\&2')} \quad (5)$$

The (6 × *integral 2′*) term represents the calculated integral value of the six protons belonging to the non-grafted GlcN units, while the *integral (2–6&2′)* term represents a sum of the integrals of all the grafted and the non-grafted GlcN units. GE is thus defined by Eq. 5:

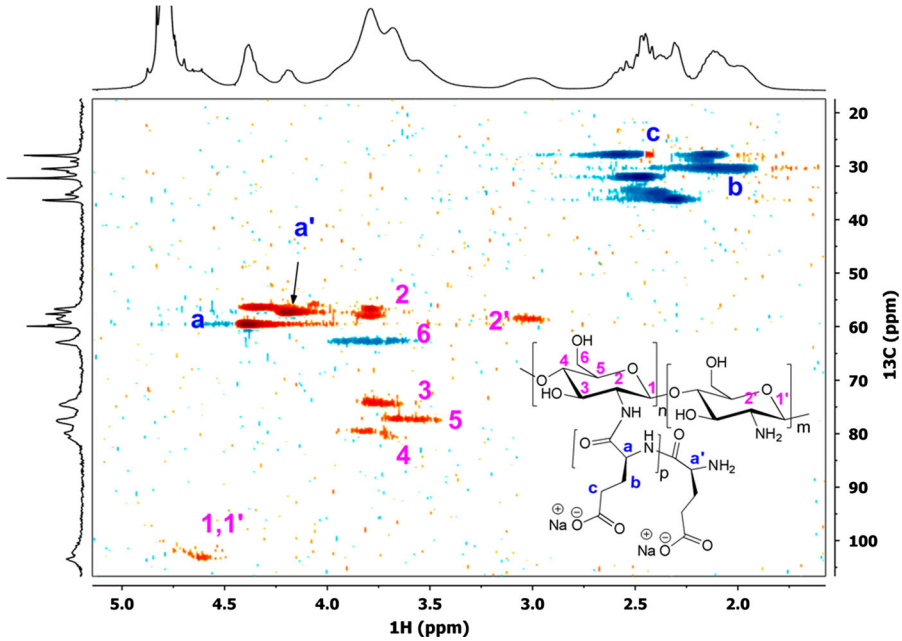

Fig. 3 gHSQCad spectrum of Chi-*g*-$PGlu_5$ in D_2O

Table 2 Glu:NH_2 ratio and GE for Chi-*g*-PGlu products estimated from their ^{1}H NMR spectra

	Integral b&c (4 protons)	Integral 2-6&2′ (6 protons)	Integral 2′	Glu:NH_2	GE
Chi-*g*-$PGlu_5$	4.0	4.90	0.57	1.2:1	0.3
Chi-*g*-$PGlu_{10}$	4.0	3.19	0.32	1.9:1	0.4
Chi-*g*-$PGlu_{15}$	4.0	2.72	0.29	2.2:1	0.4

$$GE = 1 - \left[\frac{6 \times integral\,2'}{integral\,(2-6\&2')}\right] \quad (6)$$

In that manner the GE was estimated to be between 0.3 and 0.4 (Table 2).

FT-IR spectra of the starting Chi material, the PGlu linear homopolymer and the Chi-*g*-PGlu graft copolymers (Online Resource 12) were recorded to obtain additional information on graft copolymers' structure. FT-IR spectrum of the Chi material (Lawrie et al. 2007) revealed a broad band at 3,362 cm^{-1} (hydroxyl and amine stretching), a band at 2,873 cm^{-1} (C–H stretching), a relatively weak band at 1,592 cm^{-1} (amine bending) and a weak, broad band at 1,378 cm^{-1} (C–H). Further, a broad and intensive band was observed at 1,029 cm^{-1} attributed to the C–O vibrations characteristic for the Chi's polysaccharide structure. FT-IR spectrum of the PGlu homopolymer revealed a broad absorption band at 3,289 cm^{-1} (N–H vibration), a band at 2,933 cm^{-1} (C–H stretching) and intensive bands at 1,644 cm^{-1} (amide I band), 1,558 cm^{-1} (amide II and carboxylate bands) and 1,403 cm^{-1} (carboxylate band). FT-IR spectra of the Chi-*g*-PGlu copolymers showed the presence of a broad band at approx. 3,290 cm^{-1} (hydroxyl and N–H vibrations), a band at approx. 2,930 cm^{-1} due to PGlu C–H stretching vibration. A shoulder at 2,873 cm^{-1} (C–H stretching) as well as broad and intensive band at approx. 1,030 cm^{-1} (C–O vibrations) revealed the presence of the Chi backbone moiety, whereas the intensive amide I, II and carboxylate bands at 1,648, 1,531 and 1,388 cm^{-1} clearly indicated the presence of the PGlu moieties in the structure of graft copolymers.

The results of SEC-MALS measurements of Chi-*g*-PGlu samples indicated lower molar mass averages of deprotected graft copolymers as compared to those of protected Chi-*g*-PBGlu samples, but higher than the supplied Chi material (Table 3). Since the reaction

Table 3 Results of SEC-MALS measurements for Chi-*g*-PGlu samples, degree of carboxyl group deprotection and reaction yields

	Estimated values[a]	SEC-MALS			Deprotection (%)	Yield (%)
	M_n (10^4 Da)	M_n (10^4 Da)	M_w (10^4 Da)	$Đ_M$		
Chi-*g*-PGlu$_5$	9.21	5.34	9.63	1.9	>99	70
Chi-*g*-PGlu$_{10}$	15.6	6.81	10.94	1.6	>99	66
Chi-*g*-PGlu$_{15}$	23.7	8.51	12.49	1.5	>99	61

[a] M_n values were calculated from the measured M_n values of Chi-*g*-PBGlu copolymers as determined by SEC-MALS (Table 1)

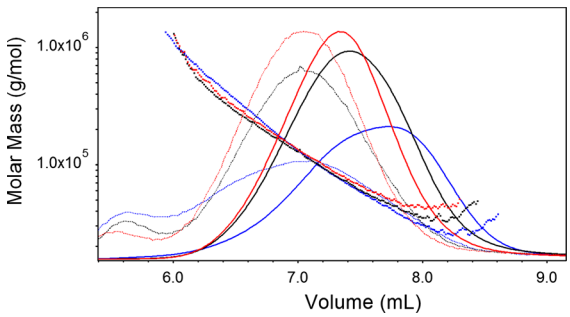

Fig. 4 SEC-MALS chromatograms together with the molar mass versus elution volume for Chi-*g*-PGlu$_5$ (*blue*), Chi-*g*-PGlu$_{10}$ (*black*) and Chi-*g*-PGlu$_{15}$ (*red*). *Solid lines*: RI responses, *dashed lines*: LS responses at angle 90°. (Color figure online)

yields (60–70 %) as well as the results of NMR spectroscopy (Glu:NH_2 ratio, Table 2) suggested partial degradation of graft copolymers during the carboxyl group deprotection with TBAH, we further wanted to determine which part of graft copolymers (Chi main chain or peptide grafts) was attacked. For this purpose, a PBGlu homopolymer was synthesized and deprotected with TBAH. Also, the Chi dispersed in water was analogously treated. Both, the PGlu and Chi treated with TBAH were analyzed by ^{1}H NMR and SEC-MALS. ^{1}H NMR spectrum of the resulting PGlu revealed complete carboxyl group deprotection, whereas the spectra of treated and untreated Chi were similar. The SEC-MALS measurements revealed lower molar mass averages of PGlu homopolymer from theoretical ones and, thus, indicated partial degradation of peptide grafts during benzyl group deprotection. On the other hand, the TBAH treated and untreated Chi showed comparable molar masses, indicating no degradation. Despite partial degradation of peptide grafts during Chi-*g*-PBGlu deprotection by TBAH, the Chi-*g*-PGlu products showed unimodal molar mass distribution with molar mass averages higher than those of the starting Chi (Fig. 4; Table 3).

Preparation and evaluation of nanoparticles of GCSF, Chi-*g*-PGlu and TMC

Preparation of complexes from GCSF and Chi-g-PGlu copolymers

The applicability of the synthesized Chi-*g*-PGlu copolymers for the preparation of NPs loaded with GCSF biopharmaceutical was tested. The buffer solution of GCSF (pH 4.5, c = 4.4 mg/mL) was added to the gently stirred aqueous solutions of the negatively charged Chi-*g*-PGlu copolymers (V = 1 mL, c = 2 mg/mL) and the formation of NPs was monitored by DLS (Table 4). Then the mixtures were centrifuged and the supernatants analyzed for the free GCSF protein content by SEC-MALS to determine the amount of GCSF not loaded into NPs (Table 4). The AE and FL were then calculated from the obtained SEC-MALS data according to Eqs. 2 and 3. In the case of Chi-*g*-PGlu$_5$ copolymer a precipitate was formed if more than 100 µL of GCSF solution was added to the graft copolymer solution and, thus, only 18 % IL was possible for this system. Beside the low IL, the AE was also very low (up to 32 %) which both resulted in FL below 6 %. In contrast to the Chi-*g*-PGlu$_5$, the Chi-*g*-PGlu$_{10}$ and the Chi-*g*-PGlu$_{15}$ copolymers proved to have much higher capacity for GCSF complexation. For the Chi-*g*-PGlu$_{10}$ and the Chi-*g*-PGlu$_{15}$ copolymers, the highest achieved IL was 52 % along with 95 and 91 % AE, respectively. Larger IL of GCSF resulted in precipitate formation. With increasing IL (40, 47 and 52 %) the AE also increased for both copolymers and, thus, led to very high FL values (up to 51 and 50 %, respectively). In addition, with increasing IL an increase in average hydrodynamic diameter

Table 4 Experimental conditions for the preparation of complexes, IL, AE and FL of GCSF protein in Chi-*g*-PGlu/GCSF complexes

Polymer[a]	GCSF[b] (μL)	Average d_H^c (nm)	PdI[d]	$GCSF^e_{(supernatant)}$ (mg/mL)	IL (%)	AE (%)	FL (%)
Chi-*g*-$PGlu_5$	80	169	0.169	0.223	15.0	31.6	5.3
	90	194	0.115	0.263	16.5	27.6	5.2
	100	230	0.155	0.289	18.0	27.8	5.8
Chi-*g*-$PGlu_{10}$	300	250	0.185	0.141	39.8	86.1	36.2
	400	290	0.204	0.081	46.8	93.6	45.2
	500	332	0.173	0.071	52.4	95.2	51.1
Chi-*g*-$PGlu_{15}$	300	238	0.121	0.291	39.8	71.3	32.0
	400	252	0.185	0.197	46.8	84.3	42.6
	500	272	0.221	0.133	52.4	90.9	50.0

[a] Chi-*g*-PGlu, c = 1 mg/mL, V = 1 mL, [b] c = 4.4 mg/mL, [c] average hydrodynamic diameter, [d] polydispersity index, [e] non-complexed GCSF content in the supernatant as determined by SEC-MALS

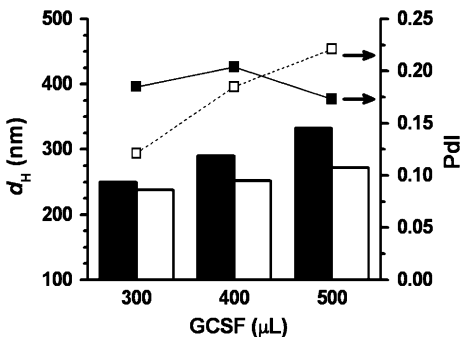

Fig. 5 A plot of the average hydrodynamic diameter (d_H, *bars*) and PdI (*squares*) versus the amount of added GCSF to the Chi-g-$PGlu_{10}$ and Chi-g-$PGlu_{15}$ copolymers solution, respectively. *Black bars* and *black squares*: Chi-*g*-$PGlu_{10}$ data. *White bars* and *white squares*: Chi-*g*-$PGlu_{15}$ data

(d_H) of NPs was observed, whereas the PdI values were maintained quite low (Table 4; Fig. 5).

Preparation of NPs from GCSF, Chi-g-PGlu copolymers and TMC

The impact of the positively charged TMC solution (c = 1 mg/mL; 30, 50 and 70 μL) added to the suspension of GCSF/Chi-*g*-PGlu complexes was also monitored by DLS measurements (Table 5). Then the suspensions were centrifuged and thus obtained supernatants analyzed by SEC-MALS for the free GCSF protein content, i.e. the amount of GCSF not loaded into NPs, in order to determine the AE and FL according to Eqs. 2 and 3. The TMC addition to the preformed GCSF/Chi-*g*-$PGlu_5$ complexes revealed low AE (below 30 %). With increasing amount of added TMC, the AE decreased most probably due to exchange of GCSF with TMC in NPs. The addition of TMC solution to GCSF/Chi-*g*-$PGlu_{10}$ complexes resulted in negligible effect on AE that was above 90 % for all the three experiments. With increasing amount of TMC added the d_H decreased from 318 nm to 295 nm and 279 nm, and the PdI decreased from 0.26 to 0.20 and 0.15 (Fig. 6). However, a comparison of the d_H and the PdI values with the corresponding values of the parent Chi-*g*-$PGlu_{10}$/GCSF complexes (Table 4; Fig. 7) revealed negligible impact of added TMC on the NPs characteristics (Tables 4, 5). The very high AE, together with high IL, resulted in very high FL, i.e. between 44 and 45 %. The TMC added to the GCSF/Chi-*g*-$PGlu_{15}$ complexes (Table 5; Fig. 6) showed almost no effect on the NPs size (d_H: from 252 nm to near 250 nm in all experiments), only the PdI (from 0.19 to approx. 0.25) and AE (from 84 to 85–89 %) somewhat increased (Fig. 7). High AE values resulted in high FL values (42–43 %). In addition, the STEM image of Chi-*g*-$PGlu_{15}$/GCSF/TMC 1/0.4/0.05 (v/v/v) NPs confirmed the average particle size determined by DLS (Fig. 7e).

Time, temperature and pH dependent stability of NPs

The time dependent stability of NPs at 25 °C was evaluated for the Chi-*g*-$PGlu_{15}$ based systems by recording the DLS measurements on fresh samples and the same samples after incubation at 25 °C for 16 days (Table 6). The Chi-*g*-$PGlu_{15}$/GCSF complexes proved to be rather unstable since both d_H

Table 5 Experimental conditions for NPs preparation, IL, AE and FL of GCSF protein in Chi-*g*-PGlu/GCSF/TMC NPs

Polymer[a]	GCSF[b] (μL)	TMC[c] (μL)	Average d_H^d (nm)	PdI[e]	GCSF$^f_{(supernatant)}$ (mg/mL)	IL (%)	AE (%)	FL (%)
Chi-*g*-PGlu$_5$	100	30	203	0.143	0.276	17.8	29.1	5.9
		50	214	0.081	0.273	17.7	28.6	5.8
		70	210	0.111	0.289	17.5	23.2	4.7
Chi-*g*-PGlu$_{10}$	400	30	318	0.259	0.068	46.4	94.5	45.0
		50	295	0.200	0.073	46.2	94.0	44.7
		70	279	0.154	0.084	46.0	93.0	44.2
Chi-*g*-PGlu$_{15}$	400	30	254	0.237	0.191	46.4	84.5	42.3
		50	254	0.259	0.173	46.2	85.7	42.4
		70	243	0.251	0.136	46.0	88.6	43.0

[a] Chi-*g*-PGlu, c = 1 mg/mL, V = 1 mL, [b] c = 4.4 mg/mL, [c] c = 1 mg/mL, [d] average hydrodynamic diameter, [e] polydispersity index, [f] non-complexed GCSF content in the supernatant as determined by SEC-MALS

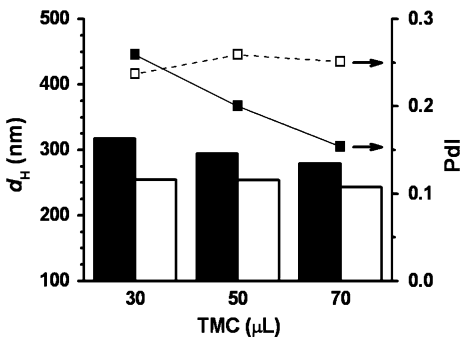

Fig. 6 A plot of the average hydrodynamic diameter (d_H, histogram) and PdI (*squares*) versus the amount of added TMC to the Chi-*g*-PGlu$_{10}$/GCSF 1/0.4 (v/v) and Chi-*g*-PGlu$_{15}$/GCSF 1/0.4 (v/v) complexes, respectively. *Black bars* and *black squares*: Chi-*g*-PGlu$_{10}$ data. *White bars* and *white squares*: Chi-*g*-PGlu$_{15}$ data

and PdI values increased after incubation to 781 nm and 0.70 as compared to the initial values of 252 nm and 0.19, respectively. The addition of TMC resulted in a less pronounced increase in the d_H and the PdI, i.e., from initial 254 nm/0.26–408 nm/0.29 after incubation.

The effect of temperature on NPs stability was evaluated in the temperature range between 25 and 39 °C with increments of 2 °C and subsequent 10 min stabilization time at each temperature before DLS measurements. The temperature stability was examined for the Chi-*g*-PGlu$_{10}$/GCSF/TMC 1/0.4/0.05 (volume ratio) and the Chi-*g*-PGlu$_{15}$/GCSF/TMC 1/0.4/0.05 (volume ratio) systems. The results are shown in the plot representing the NPs d_H values versus temperature (Fig. 8). For both systems, a slight increase in d_H with 5 and 12 % increments for the Chi-*g*-PGlu$_{10}$/GCSF/TMC and the Chi-*g*-PGlu$_{15}$/GCSF/TMC, respectively, was observed with increasing temperature due to thermal induced swelling of NPs. These results indicate thermally rather stable NPs suspensions in the temperature range investigated.

The effect of pH on the stability of the Chi-*g*-PGlu$_{10}$/GCSF/TMC and the Chi-*g*-PGlu$_{15}$/GCSF/TMC suspensions was examined by monitoring the d_H, PdI, and Zeta potential during the titration with 0.1 M NaOH solution. Zeta potential decreased from −14.5 mV for the starting Chi-*g*-PGlu$_{10}$/GCSF/TMC dispersion at pH 4.7 to the value of −27.4 mV for the dispersion with pH of 8.4. Similarly, the Zeta potential for the Chi-*g*-PGlu$_{15}$/GCSF/TMC suspension decreased from −5.4 mV at pH 4.9 to −28.1 mV at pH 8.3. Since Zeta potential of suspension decreased with increasing pH, the electrostatic repulsions between negative charges weaken the attraction forces between the associated components, which results in NPs destabilization, fragmentation and formation of smaller NPs with an average diameter below 100 nm and, consequently, in bimodal size distribution profile with significantly enlarged PdI values (Fig. 9).

Conclusion

Chi CSA, which is soluble in DMSO, was effectively applied as a macroinitiator for the ROP of BGlu NCA to prepare Chi-*g*-PBGlu graft copolymers with various lengths of the grafted polypeptide chains. Chi-*g*-

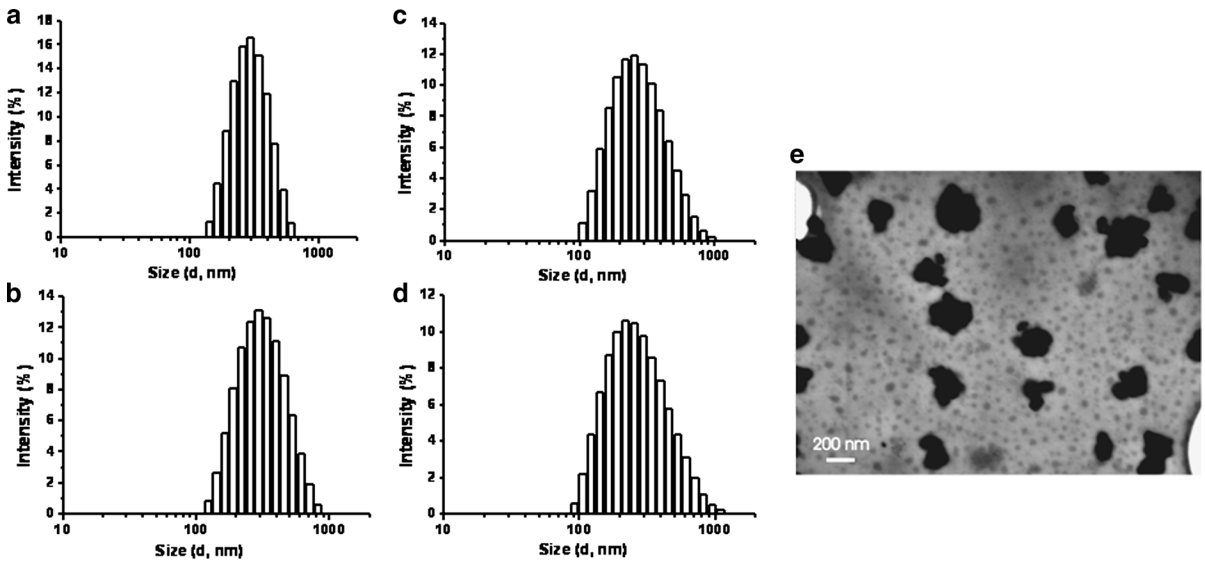

Fig. 7 DLS size distributions plots of **a** Chi-*g*-PGlu$_{10}$/GCSF 1/0.4 (v/v), **b** Chi-*g*-PGlu$_{10}$/GCSF/TMC 1/0.4/0.05 (v/v/v), **c** Chi-*g*-PGlu$_{15}$/GCSF 1/0.4 (v/v) and **d** Chi-*g*-PGlu$_{15}$/GCSF/TMC 1/0.4/0.05 (v/v/v). **e** STEM image of Chi-*g*-PGlu$_{15}$/GCSF/TMC 1/0.4/0.05 (v/v/v) NPs with average $d_H = 254$ nm as determined by DLS

Table 6 Experimental conditions for NPs preparation and DLS analysis of the NPs' time dependant stability

	GCSF[b] (μL)	TMC[c] (μL)	t_0		t_1^f	
			Average d_H^d (nm)	PdI[e]	Average d_H^d (nm)	PdI[e]
Chi-*g*-PGlu$^a_{15}$	400	0	252	0.185	781	0.696
		50	254	0.259	408	0.287

[a] c = 1 mg/mL, V = 1 mL, [b] c = 4.4 mg/mL, [c] c = 1 mg/mL, [d] average hydrodynamic diameter, [e] polydispersity index, [f] data obtained after incubation for 16 days at 25 °C

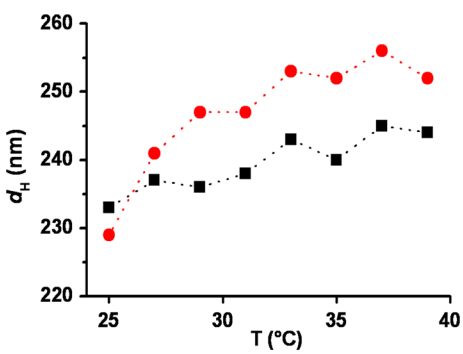

Fig. 8 A plot of the average hydrodynamic diameter (d_H) versus temperature (T) for Chi-*g*-PGlu$_{10}$/GCSF/TMC 1/0.4/0.05 (v/v/v), *black squares* and for Chi-*g*-PGlu$_{15}$/GCSF/TMC 1/0.4/0.05 (v/v/v), *red circles*. (Color figure online)

PBGlu graft copolymers were quickly and effectively deprotected by applying quaternary ammonium base TBAH. The final deprotected Chi-*g*-PGlu products exhibit number-average molar-masses between 5.3×10^4 and 8.5×10^4 Da and weight-average molar-masses between 9.6×10^4 and 12.5×10^4 Da. The results of 1D and 2D NMR experiments, FT-IR and SEC-MALS confirm the successful synthesis of the grafted copolymers. The synthesized Chi-*g*-PGlu copolymers were used together with TMC for preparation of NPs loaded with GCSF biopharmaceutical by polyelectrolyte complexation method. The influence of chemical composition of graft copolymers as well as the relative amounts of GCSF and TMC on NPs formation was investigated. The results prove that NPs based on the Chi-*g*-PGlu$_{10}$ and the Chi-*g*-PGlu$_{15}$ show very high IL capacities for GCSF protein (up to 46 %) as well as very high AE (up to 95 and 89 %, respectively). Very high IL and AE values further lead to high FL values of up to 45 and 43 %, respectively. The GCSF loaded

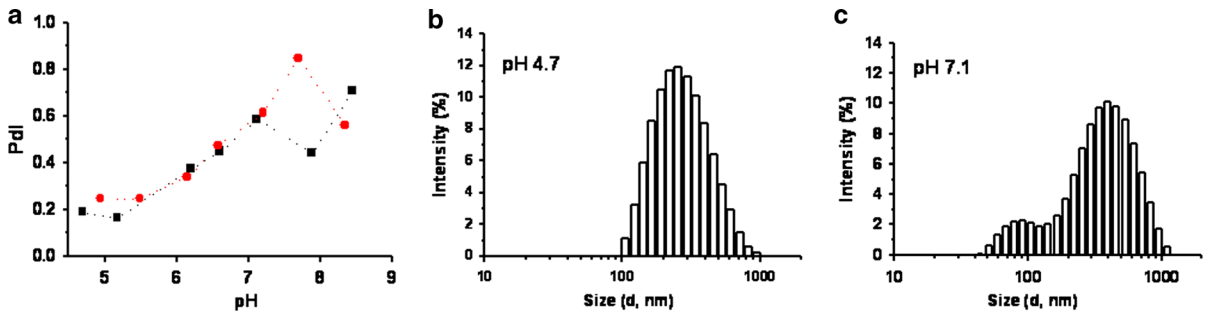

Fig. 9 **a** PdI as a function of pH for the samples Chi-*g*-$PGlu_{10}$/GCSF/TMC 1/0.4/0.05 (v/v/v), *black squares* and for Chi-*g*-$PGlu_{15}$/GCSF/TMC 1/0.4/0.05 (v/v/v), *red circles*. **b** DLS size distribution histogram of Chi-*g*-$PGlu_{10}$/GCSF/TMC 1/0.4/0.05 (v/v/v) at pH 4.7 and **c** DLS size distribution histogram for the same sample at pH 7.1. (Color figure online)

NPs were investigated in terms of thermal, pH and time dependant stability. The NPs are thermally stable in temperature range 25–39 °C and show the pH dependent destabilization at pH values near and above GCSF pI value. TMC addition has a great impact on time dependant stability of the examined NPs since its addition results in less changed NPs size and polydispersity during 16 days incubation at 25 °C as compared to the unstable GCSF/Chi-*g*-PGlu complexes without added TMC.

Acknowledgments The authors gratefully acknowledge the financial support of the Ministry of Higher Education, Science and Technology of the Republic of Slovenia through the Slovenian Research Agency (Program P2-0145 and project L2-4166).

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