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Toll-like receptor-2 agonist functionalized biopolymer for mucosal vaccination

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ABSTRACT

The objective of this study was to provide a new water-soluble chitosan derivative being functionalized with a Toll-like receptor-2 (TLR-2) agonist. At first, we synthesized the water-soluble TLR-2 agonist *ω-amido-[N_α-palmitoyl-oxy-S-[2,3-bis(palmitoyl-oxy)-(2R)-propyl]-[R]–cysteinyl]-α-amino poly(ethylene glycol)* (*Pam_3Cys-PEG-NH_2*), which was characterized by ^1H and ^{13}C NMR as well as mass spectroscopy. Secondly, *Pam_3Cys-PEG-NH_2* was then successfully grafted to 6-*O*-carboxymethyl-*N*,*N*,*N*-trimethyl chitosan polymers (*CM-TMC*) using EDC/NHS as condensing agents. The copolymer was analysed by means of ^1H and ^{13}C NMR and FTIR spectroscopy. ^{13}C NMR spectroscopy did not deliver evidence that an amide bond was formed between *CM-TMC* and *Pam_3Cys-PEG-NH_2*. However, ^1H NMR and FTIR spectroscopy demonstrated clearly that successful grafting took place. Based upon these results, this new TLR-2 functionalized biopolymer merits further investigations as material for vaccine delivery systems.

1. Introduction

Chitosan is a linear, cationic polysaccharide consisting of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit) monomers. It is industrially produced by alkaline deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans. Due to many advantageous properties, such as low toxicity, absorption enhancement of hydrophilic drugs and mucoadhesive properties (van der Lubben et al., 2001a; Chopra et al., 2006), chitosan attracted considerable attention as a novel excipient in mucosal drug and vaccine delivery. Regarding the latter, chitosan polymers can easily encapsulate or adsorb antigens via the formation of micro- or nanoparticles, which has been demonstrated, e.g., for tetanus toxoid, diphteria toxoid (van der Lubben et al., 2001b) and a plasmid DNA encoding eight different antigenic epitopes of *M. tuberculosis* (Bivas-Benita et al., 2004).

However, the majority of such vaccines lack most of the features of the original pathogen, such as innate immune stimulation, and are therefore often poorly immunogenic (OïHagan et al., 2006). Hence, non-specific stimulators of the immune system (adjuvants) are needed to render a vaccine more effective in view of eliciting protective immunity (Pashine et al., 2005).

The present strategy for the development of new vaccines is to include highly purified synthetic adjuvants, which are able to trigger well-defined elements of the immune system.

Hereby, so-called Toll-like receptor (TLR) agonists have been lately considered as very auspicious due to their ability to elicit a significant innate immune response which, in turn, affects strongly the initiation of adaptive immunity (Iwasaki and Medzhitov, 2004).

TLRs are a family of at least 10 receptors able to recognize and discriminate highly conserved microbial structural motives of bacteria, viruses, fungi and protozoae. Their activation results in an immune response accompanied by increased levels of pro-inflammatory and immune-related cytokines. Maturation of dendritic cells and their subsequent migration to regional lymph nodes followed by a facilitated presentation of antigens to T-lymphocytes have been described (Iwasaki and Medzhitov, 2004).

Among all TLRs, TLR-2 recognizes the broadest repertoire of pathogen-associated molecular patterns (PAMPs) from a large variety of pathogens. TLR-2 is highly expressed on the membrane of dendritic cells, which are considered as the most potent cell type for antigen presentation (Wetzler, 2003; Iwasaki and Medzhitov, 2004; Schmitt et al., 2008).

TLR-2 recognizes its ligands as heterodimer either in combination with TLR-1 or TLR-6. A major difference between both heterodimer types is that TLR-1/TLR-2 enables recognition of triacylated lipoproteins, whereas TLR-2/TLR-6 detects diacylated lipoproteins and peptidoglycans (Wetzler, 2003).

Notably, *N_α-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-Cys-[S]-Ser-[S]-Lys (4) trihydrochloride* (*Pam_3CSK4*), a synthesized tripalmitoylated lipopeptide, is capable to trigger

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TLR-2 as a TLR-1/TLR-2 agonist and possesses a strong adjuvant capacity (Lombardi et al., 2008; Wedlock et al., 2008).

Moreover, Kleine et al. demonstrated that conjugates of the lipophilic *Pam$_3$Cys* moiety coupled to poly(ethylene glycol) (PEG) become water-soluble (up to 10 mg/mL), while retaining their immunological properties (Kleine et al., 1994).

In order to combine the adjuvant capacity of *Pam$_3$Cys* moiety with the excellent mucosal vaccine delivery features of chitosan polymers, we synthesized the pure diastereomeric TLR-2 agonist *ω-amido-[N$_α$-palmitoyl-oxy-S-[2,3-bis(palmitoyl-oxy)-(2R)-propyl]-[R]–cysteinyl]-α-amino poly(ethylene glycol)* (abbreviated *Pam$_3$Cys-PEG-NH$_2$*, molecular weight ~3990 Da) and coupled it covalently to a new water-soluble chitosan polymer. This concept would allow an ideal combination of adjuvant and (encapsulated or surface adsorbed) antigens in the same particulate system (chitosan polymer), which is supposed to be required for effective vaccines (OïHagan et al., 2006; Schlosser et al., 2008).

As water-soluble chitosan derivative, we selected 6-*O*-carboxymethyl-*N*,*N*,*N*-trimethyl chitosan polymer (*CM-TMC*, molecular weight ~200 kDa) and applied a relatively low grafting ratio (*Pam$_3$Cys-PEG-NH$_2$*: *CM-TMC*) of ≤5% so that the main characteristics of chitosan as polymeric delivery system were preserved.

In order to synthesize *CM-TMC* we started firstly by trimethylation of chitosan polymer giving *N*,*N*,*N*-trimethyl chitosan polymer (*TMC*). This chitosan derivative demonstrated beneficial water-solubility at physiological pH values due to its permanent quaternization (Sahni et al., 2008) and featured strong adjuvant properties when used as vaccine delivery system, e.g., for a CRM–MenC conjugate vaccine (Baudner et al., 2004, 2005).

The synthesis of *CM-TMC* from *TMC* was described previously by Murata and colleagues, however, neither the molecular weight of chitosan (e.g., oligomeric or polymeric) used and conditions under which the chemical synthesis was performed were mentioned (Murata et al., 1996, 1997).

Furthermore, Jansma et al. (2003) synthesized 6-*O*-carboxymethyl-*N*,*N*,*N*-trimethyl chitosan oligomers (*CM-TMO*). However, oligomeric chitosan is readily water-soluble, whereas polymeric chitosan solely dissolves in diluted acidic aqueous solutions (pH < 6.5) (Qin et al., 2006).

The aim of our study was to establish a reproducible method for the synthesis of *CM-TMC* and to successively graft *Pam$_3$Cys-PEG-NH$_2$* to polymeric *CM-TMC*.

2. Materials and methods

2.1. Materials

Chitosan (ChitoClear® cg110), of an intrinsic viscosity of 33 mPa s, a molecular weight (MW) of approximately 200 kDa, and a degree of deacetlyation (DD) of 94% was purchased from Primex, Iceland. Dialysis membrane Spectra/Por 4 (cut-off 12–14 kDa) was obtained from Spectrum, USA. *N*,*N*′-Bis(fluorenylmethoxycarbonyl)-[*R*]-cystine-bis-*tert*-butyl ester was purchased from Bachem, Switzerland. *α*,*ω*-Bis-amino poly(ethylene glycol), PEG diamine (MW ~ 3000 Da) was purchased from Iris Biotech GmbH, Germany. All the other reagents and solvents were of analytical grade and supplied by Sigma–Aldrich, Switzerland.

2.2. Characterization of polymers

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian VXR 300 MHz or 500 MHz spectrometer (Varian, Switzerland). Chitosan was dissolved in 1% (v/v) DCl/D$_2$O, chitosan derivatives in D$_2$O and all other compounds in CDCl$_3$. Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me$_4$Si). MS spectra were recorded on an API 150 EX LC/MS System (Applied Biosystems/MDS Sciex, Switzerland) equipped with a turbo ion spray ionization source. The MALDI-Tof mass spectrometry was carried out on an Axima CFR$^+$, Shimadzu mass spectrometer, using 2-(4-hydroxyphenylazo)-benzoic acid (HABA) as matrix. Homogeneity was confirmed by thin layer chromatography (TLC) on silica gel Merck 60 F254 aluminium-backed plates. Solutions were routinely dried over anhydrous sodium sulphate prior to evaporation. Chromatographic purifications were performed by employing a Merck 60 70–230 and 230–400 mesh ASTM silica gel column. FTIR spectra were recorded on a Perkin-Elmer 100 FT-IR spectrometer (PerkinElmer, Switzerland) in the range of 4000–400 cm^{-1} using KBr pellets (1%, w/w of product in KBr).

2.3. Synthesis of TMC

Trimethylation of chitosan polymers was performed according to a previously published method (Sieval et al., 1998) that was slightly modified. Briefly, chitosan polymer (2 g) was suspended in 1-methyl-2-pyrrolidinone (NMP, 80 mL) with sodium hydroxide (NaOH, 4.8 g) at 60 °C under stirring in a round bottom flask. Sufficient NaOH (11 mL of 15%, w/v; aqueous) was added in order to maintain an alkaline environment throughout the reaction.

Methylation was achieved through nucleophilic substitution by the addition of methyl iodide (MeI, 12 mL). 70 min later, the solution was taken and products were precipitated by the addition of sufficient volumes of a mixture of diethyl ether and ethanol (ratio of 1:1, v/v). The wet product was again dissolved in 80 mL NMP at 60 °C and 10 mL of 15% (w/v) sodium hydroxide (NaOH, aqueous) was added to the solution. After addition of 7 mL methyl iodide, the reaction was let under condensor for 35 min. Next, 0.6 g NaOH pellets and 5 mL MeI were added for further 35 min. *TMC* was then precipitated by using 5–10 volumes diethyl ether:ethanol (1:1, v/v) and centrifuged (1850 × *g*, 15 min). The finalized product was subsequently dissolved in 40 mL 10% NaCl solution to perform counter ion replacement and to prevent iodine oxidation. After stirring overnight the *TMC* solution was dialysed over 3 days (twice daily changing deionized water) and finally freeze-dried. The degree of trimethylation (DTM), dimethylation (DDM), 3-hydroxy- (D3OM) and 6-hydroxy-methylation (D6OM) were calculated as reported elsewhere (Polnok et al., 2004; Verheul et al., 2008). Yield 36%. ^{1}H NMR (300 MHz, D$_2$O): δ 2.0 (COCH$_3$); δ 2.8 (N–(CH$_3$)$_2$); δ 3.3 (N$^+$–(CH$_3$)$_3$); δ 3.4 (6O–CH$_3$); δ 3.5 (3O–CH$_3$); 4.8–6.0 (H-1, CH). DTM 61.4%; DDM 35.8%; DD 3.8%; D3OM 21.3%; D6OM 22.1%. ^{13}C NMR (300 MHz, D$_2$O): δ 42.1 (N–(CH$_3$)$_2$); δ 54.5 (N$^+$–(CH$_3$)$_3$); δ 58.9 (C2); δ 61.1 (C6); δ 77.3 (C3); δ 96.7 (C1). FTIR: 3429 (O–H stretching); 2925 (C–H asymmetric stretching of methyl groups); 1642 (C=O stretching of amide groups); 1478 (C–H asymmetric bending of methyl groups); 1076 (C–N stretching of primary amine groups).

2.4. Synthesis of CM-TMC

6-*O*-Carboxymethylation of *TMC* was performed pursuant to a modification of a method published by Jansma et al. (2003). *TMC* (0.5 g) was suspended in 50 mL of NMP and let stirring overnight at room temperature. The next day, the pH was readjusted to 10 by using 15% aqueous NaOH solution and sucessively 2.2 g of chloroacetic acid (20 mol equivalents (mol equiv.) to *TMC* sugar monomers) were added. The pH was maintained during the reaction at a value of 10 by sufficient addition of 15% aqueous NaOH solution. After 3 h, the product was precipitated by adding 5–10 volumes ethanol and diethylether (1:1, v/v) and subsequently isolated

by centrifugation (1850 × *g*, 15 min). After re-dissolution in 50 mL of MilliQ water, CM-TMC were rendered water-soluble by adjusting the pH to 5, the solution dialysed over the course of 3 days (twice daily changing deionized water) and sterile filtrated before lyophilization.

The degree of carboxymethylation (DCM) of *CM-TMC* can be estimated using the following equation:

$$\%\mathrm{DCM} = \left(\frac{[(\mathrm{CH_2})\text{–CO}]}{[\mathrm{H}]}\frac{1}{2}\right) \times 100$$

Hereby [(CH_2)–CO)] is the integral value of the methylene group of newly introduced carboxymethyl function at 4.1 ppm and again [H] the integral value of the H-1 peaks between 4.7 ppm and 6.0 ppm.

Yield 73%. ^{1}H NMR (300 MHz, D_2O): δ 2.0 ($COCH_3$); δ 2.8 (N–(CH_3)$_2$; δ 3.3 (N^+–(CH_3)$_3$); δ 3.4 (6O–CH_3); δ 3.5 (3O–CH_3); δ 4.1 (CH_2–CO); δ 4.8–6.0 (H-1, CH). DCM 17.2%; DTM 61.4%; DDM 35.8%; DD 3.8%; D3OM 21.3%; D6OM 22.1%. ^{13}C NMR (300 MHz, D_2O): δ 42.1 (N–(CH_3)$_2$); δ 54.5 (N^+–(CH_3)$_3$); δ 58.9 (C2); δ 61.1 (C6); δ 77.3 (C3); δ 96. 7 (C1); δ 175.2 (C=O of CH_2–COOH). FTIR: 1725 (C=O stretching of COOH group); 1606 (C=O asymmetric stretch vibration); 1384 (C=O symmetric stretch vibration).

*2.5. Synthesis of ω-amido-[N_α-palmitoyl-oxy-S-[2,3-bis(palmitoyl-oxy)-(2R)-propyl]-[R]–cysteinyl]-α-amino poly(ethylene glycol) (abbreviated Pam_3Cys-PEG-NH_2, **7**)*

*2.5.1. N_α-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxy-(2R)-propyl]-[R]-cysteine tert-butyl ester (**2**)*

To a solution of **1** (2.58 g; 3.34 mmol) in CH_2Cl_2 (20.4 mL), zinc (1.48 g) and a freshly prepared mixture of MeOH, 32% HCl (d = 1.16 g/mL) and concentrated H_2SO_4 (10.78 mL; 100:7:1) were added under vigorous stirring. After 15 min (S)-(−)-glycidol (1.16 mL; 32.42 mmol) was added. The mixture was stirred for 5 h at 40 °C. The solution was evaporated to about half of its original volume and diluted with 5% $KHSO_4$ (26 mL). This mixture was kept at −4 °C for 16 h and then extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4, evaporated to dryness and the crude residue chromatographed on silica gel with $CHCl_3$, then $CHCl_3$/MeOH (10:1) as eluant to yield **2** (2.74 g; 5.79 mmol) as a colourless oil. Yield 89%. R_f = 0.11, $CHCl_3$; 0.73, $CHCl_3$/MeOH (10:1). ^{13}C NMR ($CDCl_3$) δ 169 (Cys-CO); δ 155.7 (Fmoc-CO); δ 143.8, 141.3, 127.7, 127, 125.2, 120 (Fmoc); δ 83.2 (tBu-C_q); δ 70.2 (S-glyceryl-CH); δ 67.3 (Fmoc-CH_2–O); δ 63.4 (S-glyceryl-CH_2–O); δ 54 (Cys-CH); δ 47.1 (Fmoc); δ 35 (Cys-CH_2); δ 34.3 (S-glyceryl-CH_2); δ 27.9 (COOtBu–CH_3).

*2.5.2. N_α-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyl-oxy)-(2R)-propyl]-[R]-cysteine tert-butyl ester (**3**)*

2 (2.73 g; 5.78 mmol), palmitic acid (Pam-OH; 4.75 g; 18.54 mmol) and *N*,*N*′-diisopropylcarbodiimide (DIC; 3.46 mL; 22.35 mmol) were dissolved in dry tetrahydrofuran (THF, 56.7 mL). *N*-Dimethylamino-pyridine (DMAP; 0.29 g; 2.37 mmol) was added, and the mixture stirred for 2 h.

After the addition of glacial acetic acid (2.3 mL) the mixture was evaporated to dryness. The residue was recrystallized from CH_2Cl_2/MeOH (1:3; 68 mL) at −20 °C. **3** (5.10 g; 5.37 mmol) was obtained as a colourless powder. Yield 93%. R_f = 0.72, $CHCl_3$. ^{13}C NMR ($CDCl_3$) δ 173.5, 173.4 (PamCO); δ 169 (Cys-CO); δ 155.7 (Fmoc-CO); δ 143.8, 141.3, 127.7, 127, 125.2, 120 (Fmoc); δ 83.2 (tBu-C_q); δ 70.2 (S-glyceryl-CH); δ 67.3 (Fmoc-CH_2–O); δ 63.4 (S-glyceryl-CH_2–O); δ 54 (Cys-CH); δ 47.1 (Fmoc); δ 35 (Cys-CH_2); δ 34.5 (Pam-CH_2); δ 34.3 (S-glyceryl-CH_2); δ 32.2, 29.9, 29.7, 29.5, (Pam-CH_2); δ 27.9 (COOtBu–CH_3); δ 25.8, 25.1, 22.9 (Pam-CH_2); δ 14.3 (Pam-CH_3). MS (API) m/z 950 M^+.

*2.5.3. S-[2,3-bis(palmitoyl-oxy)-(2R)-propyl]-[R]-cysteine tert-butyl ester (**4**)*

To a solution of **3** (5.07 g; 5.34 mmol) in dry CH_2Cl_2 at 0 °C was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). After 20 min the mixture was dried under vacuum and chromatographed on silica gel, using as eluant $CHCl_3$ then $CHCl_3$/MeOH (95:5) to give **4** (3.45 g; 4.73 mmol). Yield 89%. R_f = 0.13, $CHCl_3$; 0.89, $CHCl_3$/MeOH (95:5). ^{13}C NMR ($CDCl_3$) δ 173.5, 173.4 (PamCO); δ 169 (Cys-CO); δ 83.2 (tBu-C_q); δ 70.2 (S-glyceryl-CH); δ 63.4 (S-glyceryl-CH_2–O); δ 54 (Cys-CH); δ 35 (Cys-CH_2); δ 34.5 (Pam-CH_2); δ 34.3 (S-glyceryl-CH_2); δ 32.2, 29.9, 29.7, 29.5 (Pam-CH_2); δ 27.9 (COOtBu–CH_3); δ 25.8, 25.1, 22.9 (Pam-CH_2); δ 14.3 (Pam-CH_3). MS (API) m/z 728 $(M-H)^+$.

*2.5.4. N_α-palmitoyl-oxy-S-[2,3-bis(palmitoyl-oxy)-(2R)-propyl]-[R]-cysteine tert-butyl ester (**5**)*

Pam-OH (1.07 g; 4.16 mmol) was activated in dry CH_2Cl_2 (62.4 mL) with DIC (0.73 mL; 4.71 mmol) and hydroxybenzotriazole hydrate (HOBt; 0.64 g; 4.71 mmol) in dry DMF at 0 °C. After 30 min **4** (3.43 g; 4.71 mmol) was added. After being stirred for 15 h at room temperature the solution was evaporated to dryness, the crude residue was dissolved in $CHCl_3$ and extracted with $NaHCO_3$ 5% and water. The organic phase was then evaporated under vacuum and crystallized from CH_2Cl_2/MeOH (1:3; 73.8 mL) at −20 °C affording **5** (2.73 g; 2.82 mmol) as a colourless powder. Yield 60%. R_f = 0.66, $CHCl_3$. ^{13}C NMR ($CDCl_3$) δ 173.6, 173.5, 173.4 (PamCO); δ 169 (Cys-CO); δ 83.2 (tBu-C_q); δ 70.2 (S-glyceryl-CH); δ 63.4 (S-glyceryl-CH_2–O); δ 54 (Cys-CH); δ 35 (Cys-CH_2); δ 34.5 (Pam-CH_2); δ 34.3 (S-glyceryl-CH_2); δ 32.2, 29.9, 29.7, 29.5 (Pam-CH_2); δ 27.9 (COOtBu-CH_3); δ 25.8, 25.1, 22.9 (Pam-CH_2); δ 14.3 (Pam-CH_3). MS (API) m/z 967 $(M-H)^+$.

*2.5.5. N_α-palmitoyl-oxy-S-[2,3-bis(palmitoyl-oxy)-(2R)-propyl]-[R]-cysteine (**6**)*

To a solution of **5** (0.57 g; 0.59 mmol) in dry CH_2Cl_2 (0.35 mL) was added trifluoroacetic acid (TFA; 0.59 mL; 7.64 mmol) and Et_3SiH (0.23 mL; 1.47 mmol). After stirring for 6 h at room temperature the solution was evaporated to dryness and the crude residue repeatedly evaporated with diethyl ether to give **6** in quantitative yield. The product was used without further purification. R_f = 0.38, $CHCl_3$/MeOH (95:5). ^{13}C NMR ($CDCl_3$) δ 174.6 (Cys-COOH); δ 173.6, 173.5, 173.4 (PamCO); δ 70.2 (S-glyceryl-CH); δ 63.4 (S-glyceryl-CH_2–O); δ 54 (Cys-CH); δ 35 (Cys-CH_2); δ 34.5 (Pam-CH_2); δ 34.3 (S-glyceryl-CH_2); δ 32.2, 29.9, 29.7, 29.5, 25.8, 25.1, 22.9 (Pam-CH_2); δ 14.3 (Pam-CH_3). MS (API) m/z 910 $(M-H)^+$.

*2.5.6. ω-amido-[N_α-palmitoyl-oxy-S-[2,3-bis(palmitoyl-oxy)-(2R)-propyl]-[R]–cysteinyl]-α-amino poly(ethylene glycol) (**7**)*

6 was activated in dry CH_2Cl_2 (4 mL) with DIC (0.06 mL; 0.4 mmol) and HOBt (0.05 g; 0.4 mmol) in dry DMF at 0 °C. After 30 min a solution of α,ω-bis-amino poly(ethylene glycol) (1.2 g; 0.4 mmol) in dry CH_2Cl_2/DMF (1:1; 4 mL) at 0 °C was added. After being stirred for 15 h at room temperature the reaction solution was evaporated to dryness, the crude residue dissolved in $CHCl_3$ and extracted with $NaHCO_3$ 5% and water. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude residue was chromatographed on silica gel (230–400 mesh) with CH_2Cl_2/MeOH/H_2O (91:9:0.1) then CH_2Cl_2/MeOH/H_2O (78:19:1) as eluant to yield **7** (0.41 g; 0.1 mmol) as a colourless powder. Yield 30%. R_f = 0.2; CH_2Cl_2/MeOH/H_2O (91:9:0.1). ^{1}H NMR (300 MHz, D_2O): δ 0.9 (CH_3-Pam); δ 1.4 (CH_2-Pam); δ 1.7 (C_{13}–CH_2); δ 2.2 (C_{14}–CH_2); $\delta \sim$ 3.6 (PEG-CH_2); δ 4.5 (S-glyceryl-CH_2–O); δ 5.0 (S-glyceryl-CH). ^{13}C NMR ($CDCl_3$) δ 173.6, 173.5, 173.4 (PamCO); δ 170.4 (CysCO); δ 74.0, 70.8, 70.5 (PEG-CH_2); δ 70.3 (S-glyceryl-CH); δ 63.8 (S-glyceryl-CH_2–O); δ 52.1 (Cys-CH); δ 39.7 (PEG-CH_2); δ 35.1 (Cys-CH_2); δ 34.6 (Pam-CH_2); δ 34.3 (S-glyceryl-CH_2); δ 32.2, 29.9, 29.7, 29.5, 25.8, 25.1, 22.9 (Pam-CH_2); δ 14.3 (Pam-CH_3).

FTIR: 3429 (O–H stretching); 2918 (C–H asymmetric stretching of methyl groups); 1725 (C=O stretching of the COOH group); 1639 (C=O stretching of amide groups); 1470 (C–H asymmetric bending of methyl groups); 1352 (C=O symmetric stretch vibration); 1102 (C–H stretching). MS (MALDI-Tof) m/z 3990 (M^+).

2.6. Grafting of Pam_3Cys-PEG-NH_2 (**7**) to CM-TMC

7 (10.6 mg; 0.002 mmol, 0.2 mol equiv. to COOH) was completely dissolved in 1 mL of MilliQ water by gently heating at 40 °C for 5 min. Separately, *CM-TMC* (degree of trimethylation 61.4%; degree of carboxymethylation (DCM) 17.2%; 20 mg; 0.089 mmol sugar units, 0.015 mmol COOH) was dissolved in MilliQ water (3 mL) at room temperature and the pH adjusted to 7 ± 0.1. Next, EDC (5.7 mg, 0.03 mmol, 2 mol equiv. to COOH) and NHS (3.5 mg; 0.03 mmol, 2 mol equiv. to COOH) were added and the pH readjusted to neutrality. The solution containing **7** was then added and reaction was performed over 96 h, whereby the pH was maintained at 7 ± 0.1. In the following, the solution was dialysed (membrane cut-off 12–14 kDa) over 1 week (medium twice daily changed), sterile filtered and then freeze-dried. The finalized product (**8**) was then used for further investigations. The degree of grafting (DG) was calculated by using the following equation:

$$\%DG = \left(\frac{[CH_2\text{–Pam}]}{[H]}\frac{1}{66}\right) \times 100$$

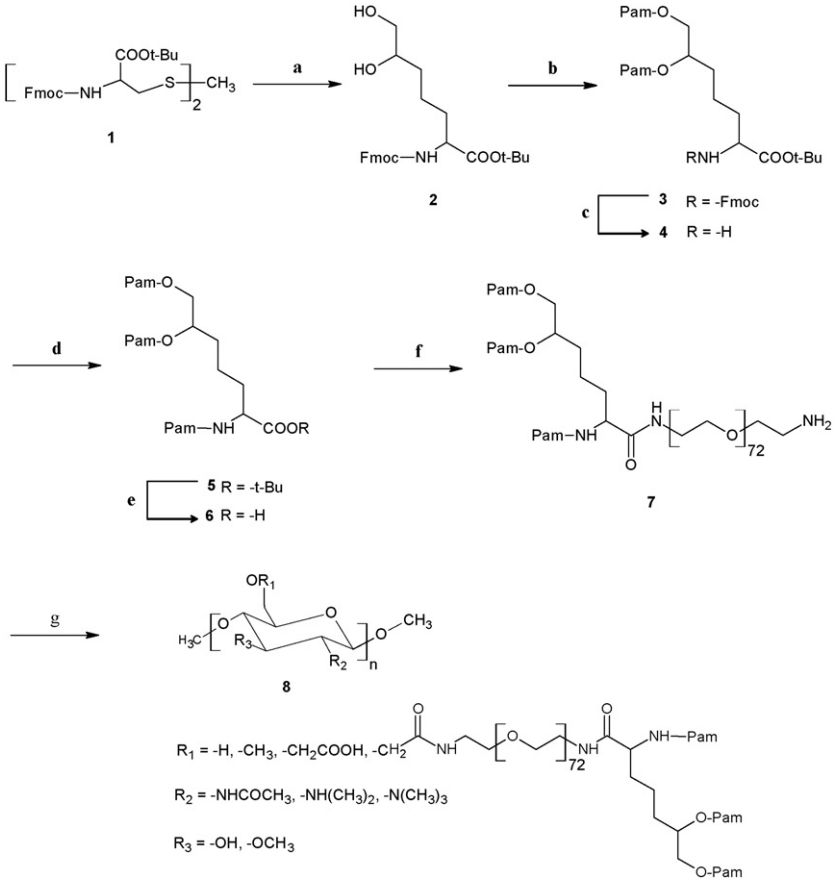
whereby [(CH_2-Pam)] is the integral value of the peak at 1.3 ppm, which is assigned to methylene groups ($3 \times 11 \times CH_2$, 66H) of the palmitoyl moiety. [H] is the integral value of H-1 peaks between 4.7 ppm and 6.0 ppm. A further approach of defining the %DG was performed as follows:

$$\%DG = \left(\frac{[\text{PEG–}CH_2CH_2]}{[H]}\frac{1}{290.9}\right) \times 100$$

Hereby, ([PEG-CH_2CH_2] is an average integral value of methylene groups of the PEG unit ($72.7 \times CH_2CH_2$, 290.9H) at ~3.6 ppm. Both methods gave in accordance a grafting degree of around 4.3%. Practical yield 87%. ^{1}H NMR (300 MHz, D_2O): δ 0.9 (CH_3-Pam); δ 1.3 (CH_2-Pam); δ 2.0 ($COCH_3$); δ 2.8 (N–(CH_3)$_2$); δ 3.3 (N^+–(CH_3)$_3$); δ 3.4 (6O–CH_3); δ 3.5 (3O–CH_3); $\delta \sim 3.6$ (CH_2–CH_2–CO); δ 4.1 (CH_2–CO); δ 4.8–6.0 (H-1, CH); ^{13}C NMR (300 MHz, D_2O): δ 22.3, 36.1 (Pam-CH_2); δ 42.1 (N–(CH_3)$_2$); δ 54.7 (N^+–(CH_3)$_3$); δ 58.9 (C2); δ 61.1 (C6); ~69.8 (PEG-CH_2CH_2); δ 77.3 (C3); δ 96. 7 (C1); δ 174.9 (C=O of COOH and CO–NH-PEG). FTIR: 1725 (C=O stretching of the COOH group); 1600 (C=O asymmetric stretch vibration); 1655 (C=O stretching of amide group); 1384 (C=O symmetric stretch vibration).

3. Results and discussion

The aim of this study was at first to provide a water-soluble chitosan derivative with auspicious properties for transmucosal vaccine delivery as well as the ability to covalently bind targeting



Reagents and conditions: **a)** CH_2Cl_2, Zn/HCl/H_2SO_4, (S)-(+)-glycidol; **b)** Anhydrous THF, Pam-OH, DIC, DMAP; **c)** Anhydrous CH_2Cl_2, DBU; **d)** Anhydrous CH_2Cl_2/DMF, Pam-OH, DIC, HOBt; **e)** Anhydrous CH_2Cl_2, TFA, Et_3SiH; **f)** Anhydrous CH_2Cl_2/DMF, PEG diamine, DIC, HOBt; **g)** H_2O, *CM20-TMC60*, EDC, NHS.

Fig. 1. Reaction scheme for the synthesis of *CM20-TMC60-g-PEG-Pam_3Cys*.

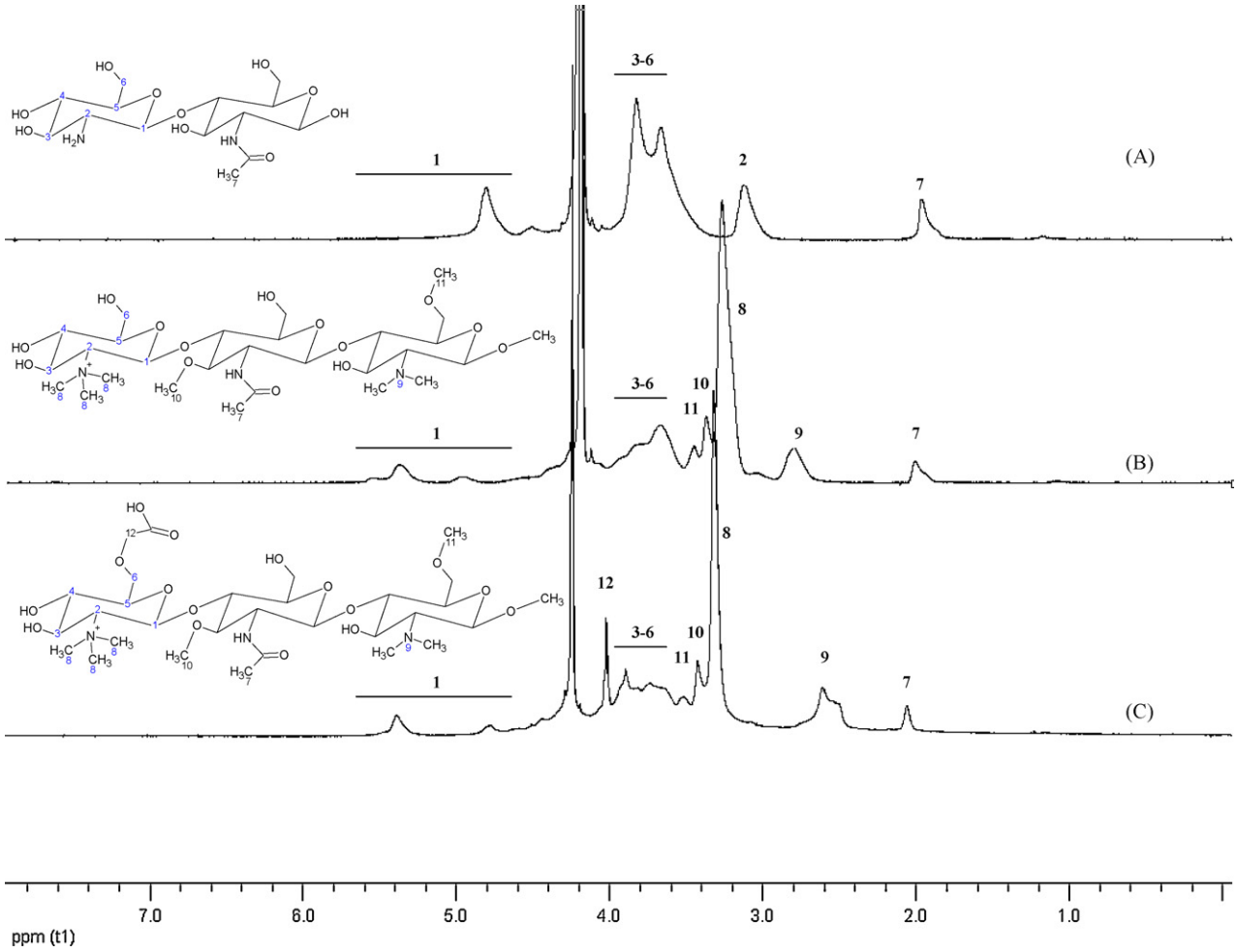


Fig. 2. ^{1}H NMR spectra of (a) chitosan (1%, v/v DCl/D_2O, 80 °C), (b) *TMC60* and (c) *CM20-TMC60* (both in D_2O at 80 °C).

ligands. We therefore opted to synthesize *TMC*, which was in turn modified to 6-*O*-carboxymethyl-*N*,*N*,*N*-trimethyl chitosan polymer (*CM-TMC*). To our best knowledge, we report here for the first time a detailed synthesis of polymeric *CM-TMC*. This new chitosan derivative may combine the promising adjuvant properties of *TMC* with the potential of covalent attachment of targeting ligands thanks to the newly introduced carboxylic functions. In a second step, we synthesized a TLR-2 targeting agonist (*Pam_3Cys-PEG-NH_2*) with the aid of modifications of already published methods (Metzger et al., 1991; Kleine et al., 1994). Finally, we grafted *Pam_3Cys-PEG-NH_2* to *CM-TMC* by the means of condensing agents (see Fig. 1).

3.1. Synthesis of TMC

Regarding the characteristics of *TMC*, the degree of trimethylation is assumed to play an important role, whereby solely *TMC* having a DTM between 40% and 60% is supposed to be beneficial as permeation enhancer of macromolecules (Sahni et al., 2008; Hamman et al., 2003). In this study, we involved a *TMC* having a DTM of around 60%, which was shown to facilitate transport of a peptide drug across the bronchial epithelium in vivo (Florea et al., 2006). Moreover, the degrees of 3- (D3OM), 6-hydroxy-methylation (D6OM) as well as dimethylation (DDM) have to be taken into consideration. As reported elsewhere, they influence cytotoxicity and physicochemical properties of *TMC* (Jintapattanakit et al., 2008; Verheul et al., 2008). A typical ^{1}H NMR spectrum of *TMC* (61.4% trimethylated, abbreviated *TMC60*) is shown in Fig. 2. Major peak assignments are as follows: $\delta = 3.3$ ppm N^{+}–$(CH_3)_3$, $\delta = 2.8$ N–$(CH_3)_2$, $\delta = 3.4$ 6O–CH_3, and $\delta = 3.5$ 3O–CH_3 (Jintapattanakit et al., 2008). In addition, the extent of di- and trimethylation of chitosan polymers was analysed by ^{13}C NMR spectroscopy, whereby the corresponding peaks appear at $\delta = 42.1$ and 54.4 ppm, respectively (data not shown; Sieval et al., 1998).

Furthermore, FTIR spectroscopy (see Fig. 3b) of *TMC60* showed the introduction of methyl groups, which can be observed at

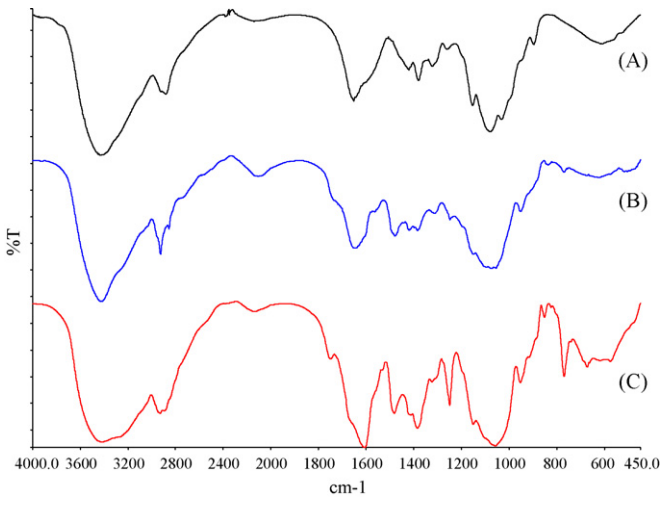


Fig. 3. FTIR spectra of (a) chitosan, (b) *TMC60* and (c) *CM20-TMC60*.

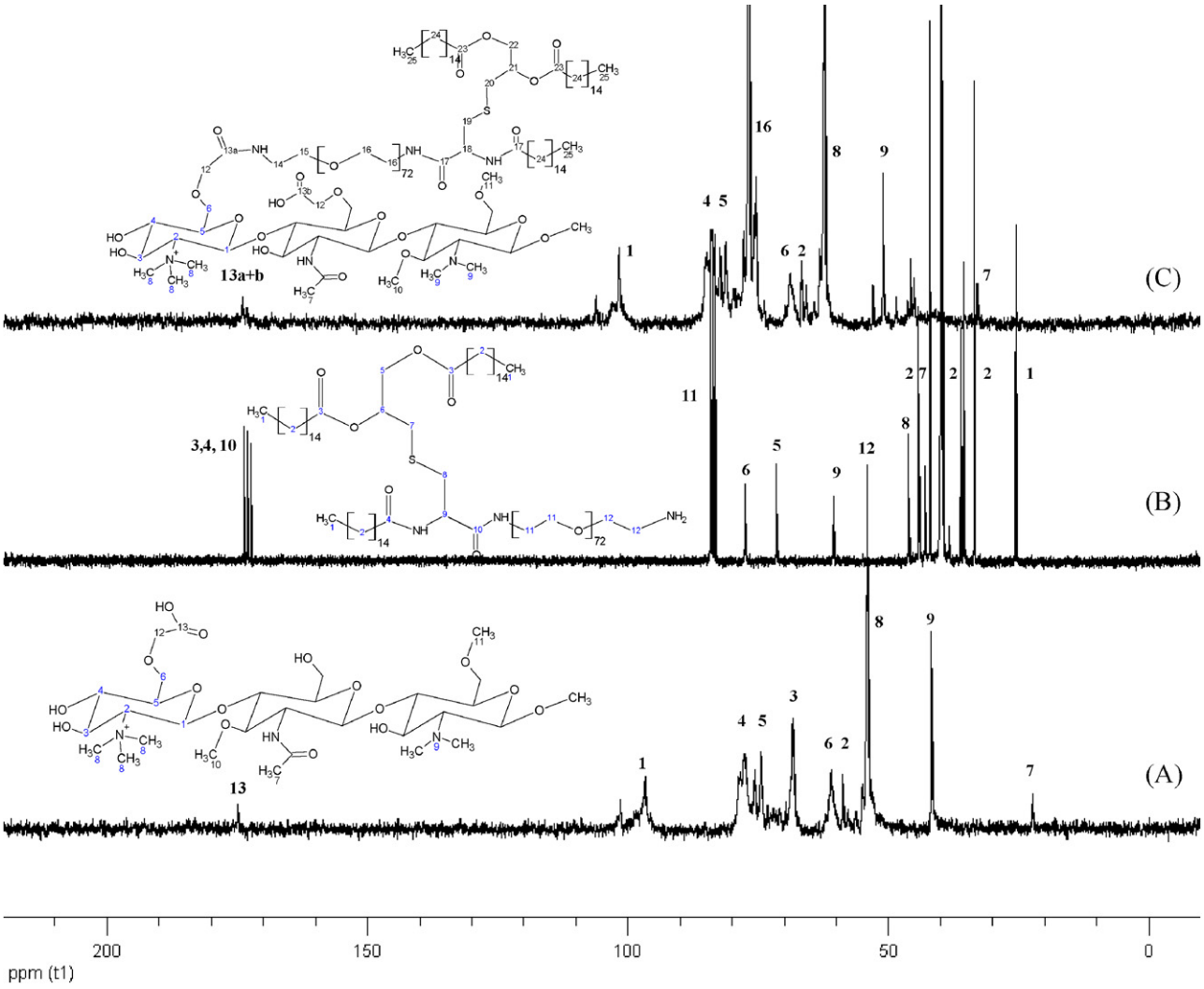


Fig. 4. ^{13}C NMR spectra of (a) *CM20-TMC60* (D_2O), (b) *Pam_3Cys-PEG-NH_2* ($CDCl_3$) and (c) copolymer *CM20-TMC60-g-PEG-Pam_3Cys* (D_2O).

2925 cm^{-1} (C–H asymmetric stretching of methyl groups) and 1478 cm^{-1} (C–H asymmetric bending of methyl groups).

3.2. Synthesis of CM-TMC

Regarding the carboxymethylation of chitosan, in most cases chloroacetic acid is used as introducing reagent. Chen and Park investigated this reaction by using chloroacetic acid in different mixtures of water and isopropanol under strong basic conditions (Chen and Park, 2003). Further on, Jansma et al. (2003) carboxymethylated trimethyl oligomers (*CM-TMO*) using chloroacetic acid in NMP at pH 10, whereby the extent of carboxymethylation was controlled by varying reaction time and equivalents of chloroacetic acid used. We opted for the latter mild method and altered it for the carboxymethylation of polymeric *TMC60*.

Fig. 2c shows the ^{1}H NMR spectrum of a *CM-TMC* with a degree of carboxymethylation (DCM) of 17.2% (abbreviated *CM20-TMC60*). The successful introduction of carboxymethyl groups can be observed at δ = 4.1 ppm (CH_2–COO). Moreover, it can be assumed that the carboxymethylation primarily occurred at the 6-*O* position due to an exclusive peak appearance at δ = 4.1 ppm (Chen and Park, 2003). Besides, the new carboxylic functionality can also be noticed in the ^{13}C NMR spectrum (see Fig. 4a) at δ 175.2 ppm (C=O of CH_2–COO). FTIR (see Fig. 3c) spectroscopy similarly indicated an effective introduction of carboxylic moieties owing to new bands at 1725 cm^{-1} (C=O stretching of COOH group); 1606 cm^{-1} (C=O asymmetric stretch vibration); 1384 cm^{-1} (C=O symmetric stretch vibration). The water-solubility of both chitosan derivatives, *TMC60* and *CM20-TMC60*, was higher than 50 mg/mL.

3.3. Synthesis Pam_3Cys-PEG-NH_2 (**7**)

The synthesis of the TLR-2 agonist **7** was performed by means of adapting already published methods (Metzger et al., 1991; Kleine et al., 1994). Compound **7** was obtained at high purity, as shown in ^{1}H NMR (Fig. 5) and MALDI-Tof spectra (Fig. 6). Moreover, the diastereomeric purity was confirmed by ^{13}C NMR spectroscopy (Fig. 4b). Finalized compound **7** was perfectly water-soluble after gently heating (40 °C for 5 min) at concentrations up to 15 mg/mL.

3.4. Grafting of Pam_3Cys-PEG-NH_2 (**7**) to CM20-TMC60

In order to enable a covalent bond between amine moieties and carboxymethylated chitosans, a number of different condensing reagents have been examined in the past. Prabaharan et al. (2007) selected 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and in a later publication a combination of EDC and *N*-hydroxysuccinimide (NHS) as condensing agents (Prabaharan and Gong, 2008), whereas Jansma et al. (2003) decided to use EDC together with *N*-hydroxybenzotriazole (HOBt). In-

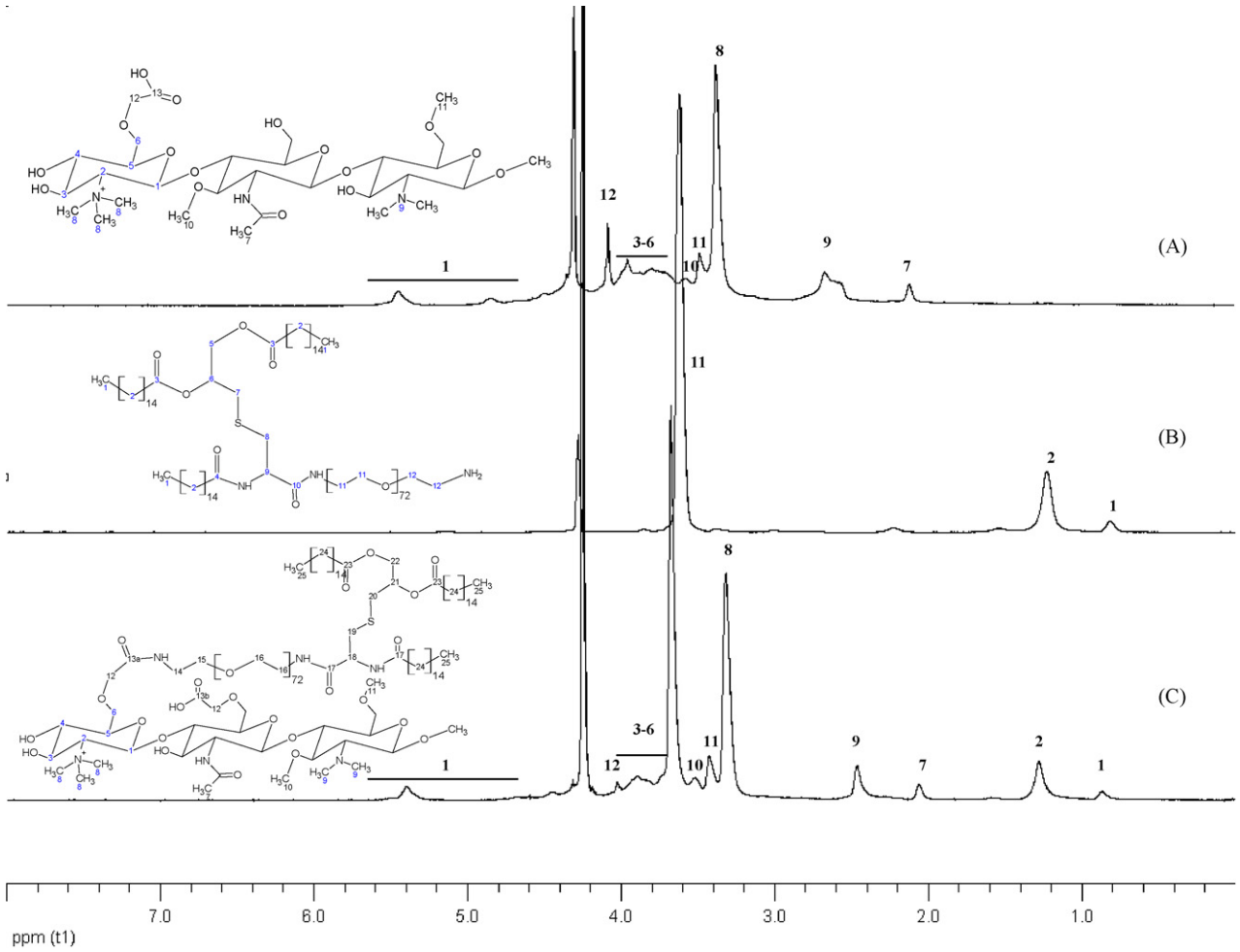


Fig. 5. ^{1}H NMR spectra of (a) *CM20-TMC60*, (b) *Pam$_3$Cys-PEG-NH$_2$* and (c) *CM20-TMC60-g-PEG-Pam$_3$Cys* (all in D_2O at 80 °C).

line with our study, we initially investigated the applicability of EDC/HOBt for grafting compound **7** to *CM20-TMC60* (data not shown). However, after purification (via dialysis) we were not able to detect any grafting of *Pam$_3$Cys-PEG-NH$_2$* to *CM20-TMC60* by NMR spectroscopy. Therefore we moved to EDC/NHS and tested their suitability as condensing agents.

In more detail, we added 2 mol equiv. (in correlation to COOH groups) of EDC and NHS to *CM20-TMC60* and let the reaction proceed in the presence of 0.2 mol equiv. (in correlation to COOH groups) of *Pam$_3$Cys-PEG-NH$_2$*, to achieve a grafting ratio of 5%. Following this method we were able to determine a grafting success (%DG) of about 4.3%, as evidenced by ^{1}H NMR spectroscopy (Fig. 5c).

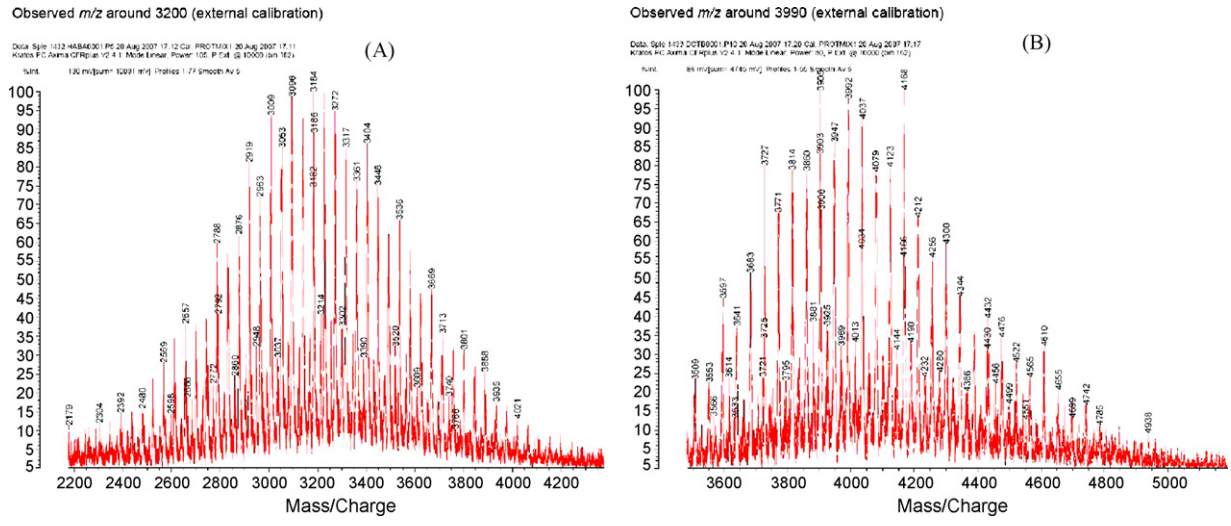


Fig. 6. MALDI-Tof spectra of (a) α,ω-bis-amino poly(ethylene glycol) (MW 3000 Da) and (b) *Pam$_3$Cys-PEG-NH$_2$* (**7**, MW 3990 Da).

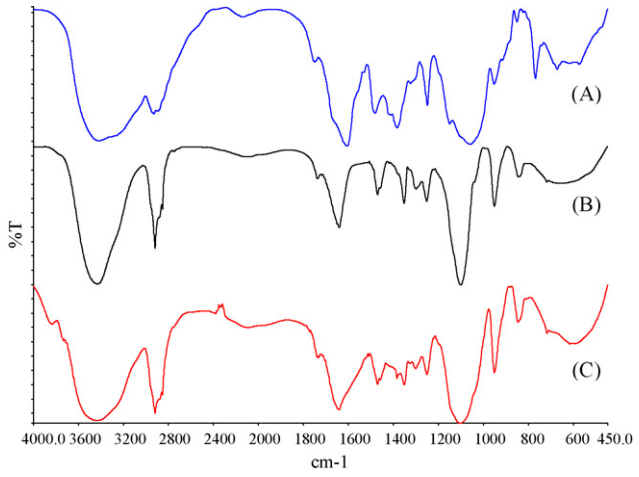


Fig. 7. FTIR spectra of (a) *CM20-TMC60*, (b) *Pam$_3$Cys-PEG-NH$_2$* and (c) *CM20-TMC60-g-PEG-Pam$_3$Cys*.

Interestingly, in this ^{1}H NMR spectrum of *CM20-TMC60-g-PEG-Pam$_3$Cys* a decrease of the peak integral at $\delta = 4.1$ ppm (CH_2–COO) was noticed. This change is likely the result of the formation of an amide bond between *CM20-TMC60* and *Pam$_3$Cys-PEG-NH$_2$*. Adjacent quantification of the DCM prior to and following the reaction depicts a decrease of 4.9%, which is in good agreement with the supposed DG of 4.3%.

In addition, the ^{13}C NMR spectrum of *CM20-TMC60-g-PEG-Pam$_3$Cys* (Fig. 4c) underlines the functionalization of the polymeric backbone of *CM20-TMC60* with the TLR-2 agonist. Although not all atom signals of *Pam$_3$Cys-PEG-NH$_2$* (Fig. 4b) were also recovered in the spectrum of the copolymer, peaks at $\delta = 22.3$ and 36.1 ppm (Pam-CH_2) as well as at ~69.8 ppm (PEG-CH_2CH_2) are pointing at an effective grafting reaction (Mao et al., 2005). Besides, the peak at around 174.9 ppm was assigned to the new amide function together with C=O peak for unreacted carboxylic moieties. This is consistent with ^{13}C NMR data reported by Jeong et al. (2008). However, Jansma et al. showed contrarily that an amide formation between oligomeric *CM-TMO* and tryptophan can be observed at 167 ppm. Considering this discrepancy together with the two facts that *Pam$_3$Cys-PEG-NH$_2$* has already three carbonylic functions by itself (Fig. 4b) and that it was solely applied at a relatively low molar ratio to *CM20-TMC60*, a clear interpretation remains difficult.

Furthermore, we analysed the copolymer with FTIR spectroscopy (Fig. 7). Here, the introduction of PEG polymer was confirmed on the basis of associated bands at 847 cm^{-1}, 950 cm^{-1} and 2917 cm^{-1} (Jeong et al., 2008). In addition, the absorption band at 1606 cm^{-1} (C=O asymmetric stretch of carboxylate anion) shifted to 1641 cm^{-1} (C=O stretch of amide), which reflects the formation of an amide bond (Prabaharan et al., 2007; Prabaharan and Gong, 2008).

4. Conclusions

Assembling all results presented, it can be concluded that the synthesis of the TLR-2 agonist was accomplished at high purity. Secondly, *Pam$_3$Cys-PEG-NH$_2$* in turn was successfully grafted to *CM20-TMC60*, although ^{13}C NMR spectroscopy did not deliver clear results regarding the formation of an amide bond. However, ^{1}H NMR and FTIR spectroscopy are of evidence of that successful grafting taking place. In conclusion, this new copolymer merits further investigations as delivery system of vaccines, such as protein or recombinant vaccines, due to its unique combination of immunomodulatory capacity and prominent mucosal vaccine delivery characteristics.

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