Conjugation of Bioactive Ligands to PEG-Grafted Chitosan at the Distal End of PEG

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Graft copolymers of chitosan and PEG−CO_2H incorporating biologically active molecules and tags (mannose, cholesterol, a coumarin dye, and biotin) at the distal end of poly(ethylene glycol) (PEG) have been synthesized in excellent yields and nearly quantitative mass recoveries. Experimental conditions allowing the preparation of multifunctional graft copolymers incorporating simultaneously several of those active molecules and tags in controlled ratios are also presented. The required functionalized PEG−CO_2H conjugates have been prepared from a heterodifunctional PEG and the experimental conditions established to ensure the purity of PEG end groups (^1H and ^{13}C NMR and matrix-assisted laser desorption/ionization mass spectrometry-time of flight (MALDI-TOF)) and the completion of each synthetic step.

Introduction

During the past decade, drug delivery systems have witnessed a revolutionary development of the so-called active targeting. This concept implies the selective and effective localization of the drug in the vicinity of the target cells in an effort to reduce toxic effects and to maximize therapeutic indexes. The most successful approach is based on the presence on the surface of a drug carrier, of ligands that specifically bind to the target cell, facilitating the trafficking of the ligand−carrier conjugate, mainly by receptor-mediated endocytosis.[1]

Similar strategies have been adopted in the design of antiadhesive therapy nanostructures. Thus, the incorporation of carbohydrates on the surface of various polymeric structures has resulted in multivalent glycoconjugates with the ability of preventing cells from pathogen and toxin invasion, metastasis, and inflammation.[2,3]

With the purpose of obtaining polymeric nanostructures with application in active targeting and antiadhesive therapy, we have turned our attention to graft copolymers of chitosan and poly(ethylene glycol) (PEG) because of their proven low toxicity and high biocompatibility (Figure 1).

Chitosan is a linear polysaccharide characterized by a high biodegradability with applications in the biomedical and pharmaceutical fields,[4] particularly in drug delivery[5] and gene transfection.[6] To further enhance the stability and biocompatibility of chitosan nanostructures in vivo, we and others have synthesized graft copolymers of chitosan with the hydrophilic polymer PEG (Figure 1).[7−10] The resulting PEG−chitosan graft copolymers (chitosan-*g*-PEG) have shown indeed improved biocompatibility[7,11] and, when engineered as nanocarriers and thermoreversible hydrogels, found application in the delivery of proteins,[12] peptides,[7,9] vaccines,[13] and nucleic acids.[14,15]

In this paper, we describe an efficient synthetic procedure for the preparation of chitosan-*g*-PEG that incorporate an array of biologically active molecules and tags useful for active targeting and antiadhesive therapy.

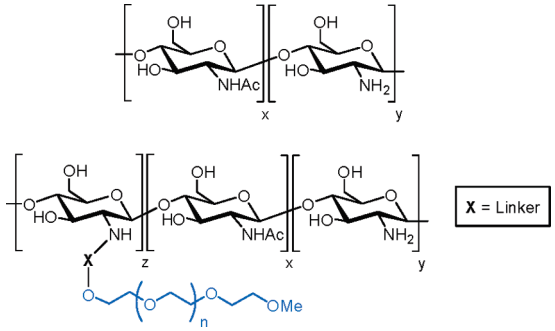

Figure 1. Chitosan and chitosan-*g*-PEG.

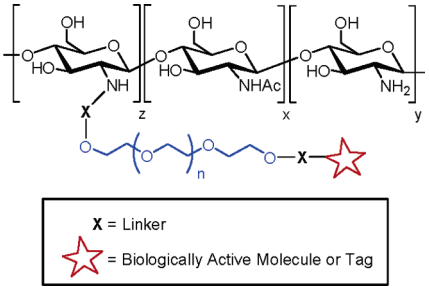

Figure 2. Chitosan-*g*-PEG functionalized at the distal end of PEG.

In our approach, the active molecules and tags have been incorporated into the distal end of PEG, instead of directly bound to the chitosan backbone,[15,16] to avoid the steric hindering of the PEG over the active molecules and to ensure an effective ligand−receptor interaction (Figure 2).[17] Moreover, such an approach minimizes the influence of the active molecules/tags on the solution properties and processing conditions of the copolymer.

With this aim, four different biologically active molecules and tags were selected on the basis of their significance from a biological or biomedical point of view: biotin (**1**), the fluorophore 7-diethylaminocoumarin-3-carboxylic acid (**2**), cholesterol (**3**), and α-D-mannose (**4**), (Figure 3). Biotin **1** was chosen because of its highly specific noncovalent interaction with avidin that has resulted in a powerful tool for assay, detection, and targeting systems.[1,18] Coumarin **2** is an interesting fluorophore

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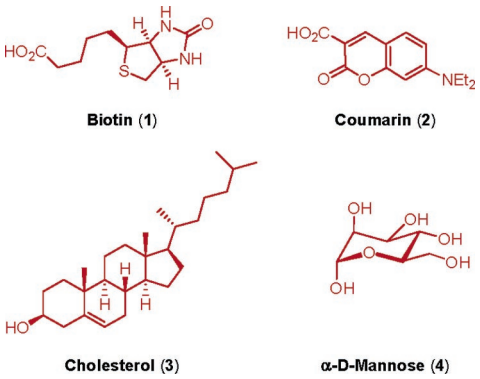


Figure 3. Active molecules and tags incorporated at the distal end of PEG.

tag excitable by visible light (410−430 nm).[19] Cholesterol **3** was selected on the basis of the recently reported increased transfection efficiency shown by hydrophobized gene carriers.[20] Finally, the presence of mannose receptors on the cells of the reticuloendothelial system, and various antigen-presenting cells, supports the selection of mannose **4**.[1,21]

Experimental Section

Materials. Ultrapure chitosan hydrochloride salt [Protasan UP CL 113, degree of acetylation (DA) 14% by ^{1}H NMR][22] was purchased from Pronova Biomedical A.S. (Norway). Heterodifunctional PEG **5** was purchased from Nektar (United States), (M_n 3837, M_w 3890, by MALDI-TOF). MeO−PEG−CO_2H **34** has been synthesized from a commercially available MeO−PEG−OH (M_n 5055, M_w 5088, by MALDI-TOF) according to known procedures.[23]

General Methods. CH_2Cl_2, $CHCl_3$, Et_3N, and pyridine were distilled from CaH_2. THF was distilled from Na/benzophenone, and MeOH was distilled from Mg. DMF was dried over 4 Å ms. Thin-layer chromatography (TLC) was done on silica 60/F-254 aluminum-backed plates. Column chromatography was performed with 70−230 and 230−400 mesh silica gel. Ultrafiltration was carried out with Amicon stirred cells (YM30 membranes). NMR spectra were recorded at Varian Inova 400 MHz and Bruker DPX 250 MHz, DPX 500 MHz, and AMX 500 MHz spectrometers in D_2O, 2% DCl in D_2O, or $CDCl_3$. Chemical shifts are reported in ppm (δ units) downfield from internal tetramethylsilane ($CDCl_3$) or 3-(trimethylsilyl)-propionic acid-d4 (D_2O).

Degree of substitution (DS) for copolymers **29**−**33** and **35** were determined by integration of the appropriate signals in the ^{1}H NMR spectra (2% DCl in D_2O, room temperature (rt)) after partially hydrolyzing the sample by heating in 2% DCl at 70 °C for 8 h. Intervals of integration: 2.00−2.20 ppm (chitosan acetyl groups) and 3.10−4.30 ppm (H2−H6 protons of chitosan, methylene protons of PEG, and protons of linkers and active molecules/tags falling in this δ range). Resonances of active molecules/tags and linkers lying out of the above intervals of integration show intensities lower than 1% of that of the PEG at 3.70 ppm and therefore have not been integrated.

SEC-MALLS. The M_w of the commercial chitosan (8.0 10^4 g/mol) was determined by size exclusion chromatography-multiangle laser-light scattering (SEC-MALLS).[24] An Iso Pump G1310A (Hewlett-Packard) was connected to a PSS Novema GPC column (10 μm, 30 Å, 8 × 300 mm, NOA0830103E1) and to a PSS Novema GPC column (10 μm, 3000 Å, 8 × 300 mm, NOA0830103E3). A PSS SLD7000 MALLS detector (Brookhaven Instruments Corporation) operating at 660 nm and a G1362A refractive index detector (Agilent) were connected online. A 0.15 M NH_4OAc/0.2 M AcOH buffer (pH = 4.5) was used as eluent. Polymer solutions were filtered through 0.2 μm pore size membranes (VWR) before injection. Polymer concentration was in the range 0.30−0.16 mg/mL. Refractive index increment dn/dC was set at 0.188, according to previous reports.[24,25]

Fluorescence. Fluorescence measurements were preformed on a fluorescence spectrophotometer SPEX Fluoromax-3. The maximum of absorbance for the PEG−coumarin conjugate **27** was shown at 427 nm (0.05 mg/mL in H_2O). Fluorescence spectra were obtained by exciting aqueous solutions of the coumarin conjugates **27**, **31A**−**31C**, and **33** at 427 nm and by recording the emission over the range 440−600 nm. The slit width was set at 2 nm for the excitation and the emission. Solutions of PEG **27** with five different concentrations ranging from $2.5\cdot10^{-2}$ to $0.5\cdot10^{-3}$ mg/mL were measured, showing a linear response. The maximum of the emission was found at 476 nm. Similarly, solutions of **31A**−**31C** and **33** with concentrations $5\cdot10^{-2}$, $5\cdot10^{-3}$, and $5\cdot10^{-4}$ mg/mL showed a maximum of emission at 476 nm.

MALDI-TOF MS. MALDI-TOF MS was carried out on a Bruker Autoflex operating in reflected mode. 2-(4-Hydroxyphenylazo)benzoic acid (HABA) was used as matrix, and NaCl or KCl was used as cationizing agent. Samples were dissolved in MeOH−H_2O (1:1) at a concentration of $5\cdot10^{-4}$ M. HABA was dissolved in dioxane at a concentration of 0.05 M. Sample (20 μL) and matrix (80 μL) solutions were mixed, and then 80 μL of 0.02 M NaCl or KCl was added. Finally, 1 μL of the resulting mixture was placed on the MALDI plate.

***N*-Boc-*N*′-biotinyl-3,6-dioxaoctane-1,8-diamine (7).** Biotin **1** (100 mg, 0.409 mmol), monoprotected diamine **6** (127 mg, 0.512 mmol, 125 mol %),[3] and HOBt (11 mg, 0.819 mmol, 200 mol %) were dissolved in DMF (6 mL) and were cooled to 0 °C under argon. EDC·HCl (157 mg, 0.819 mmol, 200 mol %) was added, and the reaction mixture was stirred for 1.5 h at 0 °C and then was allowed to reach rt. After 12 h of stirring, the solvent was evaporated, and the resulting yellow oil was purified by column chromatography (gradient from EtOAc/MeOH 5% to CH_2Cl_2/MeOH 10%) to afford **7** (181 mg, 93%) as white solid. NMR spectral data of **7** matched well those previously reported.[3]

***N*-Biotinyl-3,6-dioxaoctane-1,8-diamine (8).** Trifluoroacetic acid (TFA) (1.5 mL) was added to a solution of the biotin derivative **7** (147 mg, 0.310 mmol) in CH_2Cl_2 (3 mL). After 20 min of stirring at rt, the solvent was evaporated, and the resulting oil was dissolved in MeOH (5 mL) and was evaporated to remove any residual TFA. This process was repeated twice again leading to **8** (182 mg, 100%) as a brown oil that was used in the next step without any further purification. NMR spectral data of **8** matched well those previously reported.[3]

Boc-Protected Coumarin 9. 7-(Diethylamino)-coumarin-3-carboxylic acid (**2**) (45 mg, 0.172 mmol) and EDC·HCl (99 mg, 0.516 mmol, 300 mol %) were added to a solution of the monoprotected diamine **6** (53 mg, 0.213 mmol, 123 mol %) in CH_2Cl_2 (3.4 mL). After 4 h of stirring at rt, the reaction was partitioned between CH_2Cl_2 and H_2O. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layer was washed with brine, was dried (Na_2SO_4), and was concentrated to give a crude product that was purified by column chromatography (gradient from CH_2Cl_2 to CH_2Cl_2/MeOH 10%) to give **9** (75 mg, 89%) as an orange solid: ^{1}H NMR (250 MHz, $CDCl_3$, 300 K) δ: 1.23 (t, J = 7.1 Hz, 6H), 1.42 (s, 9H), 3.30 (dd, J = 5.1 Hz, J = 10.3 Hz, 2H), 3.44 (q, J = 7.1 Hz, 4H), 3.57 (t, J = 5.1 Hz, 2H), 3.61−3.71 (m, 8H), 5.24 (br s, 1H), 6.49 (d, J = 2.3 Hz, 1H), 6.63 (dd, J = 2.1 Hz, J = 8.9 Hz, 1H), 7.42 (d, J = 9.0 Hz, 1H), 8.69 (s, 1H), 9.05 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, 300 K) δ: 12.4, 28.4, 39.4, 40.4, 45.0, 69.8, 70.2, 70.3, 70.4, 96.6, 108.3, 109.9, 110.4, 131.0, 148.0, 152.5, 157.6, 162.6, 163.3; ESI-TOF MS m/z: found 514.2524 [M + Na]$^+$ calcd for C25H37N3O7Na: 514.2529.

Coumarin 10. From a solution of coumarin **9** (53 mg, 0.11 mmol) in CH_2Cl_2 (1 mL) and TFA (0.5 mL), and following the same procedure as for the synthesis of **8**, deprotected **10** (65 mg, 96%) was obtained as an orange solid that was used in the next step without any further purification: ^{1}H NMR (250 MHz, $CDCl_3$, 300 K) δ: 1.23 (t, J = 7.1 Hz, 6H), 3.30 (dd, J = 5.1 Hz, J = 10.3 Hz, 2H), 3.44 (q, J = 7.1 Hz, 4H), 3.61−3.71 (m, 8H), 3.77 (s, 2H), 6.49 (d, J = 2.3 Hz, 1H), 6.63 (dd, J = 2.1 Hz, J = 8.9 Hz, 1H), 7.42 (d, J = 9.0 Hz, 1H), 7.57 (br s, 1H), 8.25 (br s, 3H), 8.69 (s, 1H), 9.05 (br s, 1H).

***N*-Boc-*N*′cholesteryl-3,6-dioxaoctane-1,8-diamine (12).** Chloroformate **11** (500 mg, 1.11 mmol) was added to a solution of monoprotected diamine **6** (346 mg, 1.39 mmol, 125 mol %) and Et_3N (230 μL, 1.67 mmol, 150 mol %) in CH_2Cl_2 (11 mL) under argon. After 12 h of stirring at rt, the reaction was partitioned between CH_2Cl_2 and 5% HCl (aq). Then, the aqueous layer was extracted with CH_2Cl_2, and the combined organic layer was washed with brine, dried (Na_2SO_4), and concentrated to give a crude product that was purified by column chromatography (EtOAc/hexane, 3/4) to give **12** (705 mg, 96%) as a colorless syrup: ^{1}H NMR (500 MHz, $CDCl_3$, 333 K) δ: 0.70 (s, 3H), 0.86 (d, J = 1.7 Hz, 3H), 0.88 (d, J = 1.7 Hz, 3H), 0.92 (d, J = 6.6 Hz, 3H), 1.01 (s, 3H), 1.02−1.42 (m, 14H), 1.43 (s, 9H), 1.45−1.63 (m, 6H), 1.75−2.05 (m, 6H), 2.24−2.33 (m, 1H), 2.37 (ddd, J = 1.9 Hz, J = 5.1 Hz, J = 13.0 Hz, 1H), 3.31 (dd, J = 3.8 Hz, J = 9.1 Hz, 2H), 3.36 (dd, J = 4.0 Hz, J = 9.3 Hz, 2H), 3.46 (dd, J = 5.0 Hz, J = 9.4 Hz, 4H), 3.52 (s, 4H), 4.50 (ddd, J = 4.6 Hz, J = 11.2 Hz, J = 16.2 Hz, 1H), 4.92 (br s, 1H), 5.02 (br s, 1H), 5.34−5.38 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$, 300 K) δ: 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 28.0, 28.1, 28.2, 28.4, 31.8, 31.9, 35.7, 36.1, 36.5, 36.9, 38.5, 39.5, 39.7, 40.2, 40.5, 42.3, 50.0, 56.1, 56.6, 70.1, 70.2, 74.3, 122.4, 139.8, 155.9, 156.1; ESI-TOF MS *m*/*z*: Found 683.4970 $[M + Na]^+$. Calcd for $C_{39}H_{68}N_2O_6Na$: 683.4974

***N*-Cholesteryl-3,6-dioxaoctane-1,8-diamine (13).** From a solution of the protected cholesterol **12** (200 mg 0.303 mmol) in CH_2Cl_2 (1.6 mL) and TFA (0.8 mL), and following the same procedure as for the synthesis of **8**, deprotected **13** (204 mg, 100%) was obtained as a brown solid that was used in the next step without any further purification: ^{1}H NMR (500 MHz, $CDCl_3$, 333 K) δ: 0.70 (s, 3H), 0.86 (d, J = 1.7 Hz, 3H), 0.88 (d, J = 1.7 Hz, 3H), 0.92 (d, J = 6.6 Hz, 3H), 1.01 (s, 3H), 1.02−1.43 (m, 14H), 1.45−1.63 (m, 6H), 1.75−2.05 (m, 6H), 2.24−2.40 (m, 2H), 3.17 (dd, J = 3.8 Hz, J = 9.1 Hz, 2H), 3.32 (br s, 2H), 3.54 (s, 2H), 3.61 (s, 2H), 3.65 (s, 2H), 3.74 (s, 2H), 4.40−4.54 (m, 1H), 5.34−5.38 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$, 333 K) δ: 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 28.0, 28.1, 28.2, 31.8, 31.9, 35.8, 36.2, 36.7, 36.9, 38.5, 39.6, 39.9, 40.1, 40.2, 41.0, 42.5, 50.3, 56.4, 56.9, 66.2, 66.4, 70.1, 70.3, 74.3, 122.9, 139.8, 158.0, 160.6.

2-[2-(2-Aminoethoxy)-ethoxy]-ethyl 2,3,4,6-tetra-O-acetyl-α-D-mannoside (15). Pd/C (11 mg, 20%) was added to a solution of mannoside **14** (78 mg, 0.154 mmol)[26] in deoxygenated MeOH (2 mL). TFA (40 μL, 500 mol %) was added, and the resulting mixture was stirred under H_2 (1 atm) for 1 h. Then, the mixture was filtered (Celite) and concentrated to give **15** (90 mg, 98%) as a brown oil that was used in the next step without any further purification: ^{1}H NMR (250 MHz, $CDCl_3$, 300 K) δ: 1.91 (s, 3H), 1.97 (s, 3H), 2.02 (s, 3H), 2.07 (s, 3H), 3.17 (br s, 2H), 3.41−3.89 (m, 10H), 3.92−4.10 (m, 2H), 4.29 (dd, J = 4.6 Hz, J = 12.1 Hz, 1H), 4.79 (br s, 1H), 5.02−5.33 (m, 2H).

Man−PEG−NHFmoc (16). Et_3N (33 μL, 0.236 mmol) was added to a solution of PEG **5** (187 mg, 0.05 mmol) and mannoside **15** (89 mg, 0.150 mmol) in CH_2Cl_2 (4.8 mL). The reaction was stirred at rt for 14 h before being evaporated. The resulting crude product was dissolved in CH_2Cl_2 (1 mL), was precipitated by the addition of Et_2O (∼200 mL), and was filtered to give **16** (202 mg, 98%) as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 333 K) δ: 1.99 (s, 3H), 2.04 (s, 3H), 2.10 (s, 3H), 2.15 (s, 3H), 2.49 (t, J = 5.8, 2H), 3.34−3.99 (m, ∼330H), 4.06 (ddd, J = 2.3 Hz, J = 4.8 Hz, J = 9.3 Hz, 1H), 4.11 (dd, J = 2.3 Hz, J = 12.3 Hz, 1H), 4.22 (t, J = 6.8 Hz, 1H), 4.29 (dd, J = 5.0 Hz, J = 12.2 Hz, 1H), 4.40 (d, J = 7.1 Hz, 2H), 4.88 (d, J = 0.9 Hz, 1H), 5.25−5.33 (m, 2H), 5.35 (dd, J = 3.3 Hz, J = 10.0 Hz, 1H), 5.55 (br s, 1H), 6.71 (br s, 1H), 7.31 (t, J = 7.4 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.61 (d, J = 7.2 Hz, 2H), 7.76 (d, J = 7.3 Hz, 2H); MALDI-TOF MS *m*/*z*: M_n 4269, M_w 4293, peak average molecular weight (M_p) 4247.8 $[M + K]^+$. Calcd: M_n 4207, M_p 4248.0 $[M + K]^+$.

Cholesterol−PEG−NHFmoc (17). From a solution of PEG **5** (100 mg, 0.026 mmol), **13** (51 mg, 0.076 mmol), and Et_3N (18 μL, 0.130 mmol) in CH_2Cl_2 (2.6 mL), and following the same procedure as for the synthesis of **16**, the cholesterol conjugate **17** (107 mg, 96%) was obtained as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 313 K) δ: 0.70 (s, 3H), 0.86 (d, J = 1.7 Hz, 3H), 0.88 (d, J = 1.7 Hz, 3H), 0.92 (d, J = 6.6 Hz, 3H), 1.01 (s, 3H), 1.02−1.43 (m, 14H), 1.45−1.63 (m, 6H), 1.75−2.05 (m, 6H), 2.24−2.40 (m, 2H), 2.43 (t, J = 5.7 Hz, 2H), 3.20−3.90 (m, ∼330H), 4.15 (t, J = 6.6 Hz, 1H), 4.40 (d, J = 6.7 Hz, 2H), 4.41−4.54 (m, 1H), 5.09 (br s, 1H), 5.34−5.38 (m, 1H), 6.57 (br s, 1H), 7.31 (t, J = 7.4 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.61 (d, J = 7.2 Hz, 2H), 7.76 (d, J = 7.3 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$, 300 K) δ: 11.7, 18.6, 19.1, 21.0, 22.5, 22.6, 23.7, 24.1, 27.8, 28.0, 28.1, 31.8, 31.9, 35.6, 36.1, 36.5, 37.0, 38.5, 39.1, 39.4, 39.7, 40.8, 41.0, 42.3, 47.4, 50.1, 56.2, 56.8, 66.2, 66.4, 67.2, 69.7, 69.8, 69.9, 70.1, 70.2, 70.5, 74.3, 119.0, 122.2, 124.8, 126.9, 127.5, 139.8, 140.2, 143.9, 156.1, 156.4, 171.2. MALDI-TOF MS *m*/*z*: M_n 4359, M_w 4385, M_p 4417.5 $[M + K]^+$. Calcd: M_n 4282, M_p 4329.4 $[M + K]^+$.

Coumarin−PEG−NHFmoc (18). From a solution of PEG **5** (119 mg, 0.031 mmol), **10** (58 mg, 0.094 mmol), and Et_3N (21 μL, 0.151 mmol) in CH_2Cl_2 (3.1 mL) protected from the light, and following the same procedure as for the synthesis of **16**, the coumarin conjugate **18** (107 mg, 96%) was obtained as an orange powder: ^{1}H NMR (300 MHz, $CDCl_3$, 300 K) δ: 1.23 (t, J = 7.1 Hz, 6H), 2.53 (t, J = 6.1 Hz, 2H), 3.20−3.90 (m, ∼330H), 4.22 (t, J = 6.8 Hz, 1H), 4.40 (d, J = 7.1 Hz, 2H), 5.50 (br s, 1H), 6.49 (d, J = 2.2 Hz, 1H), 6.65 (dd, J = 2.4 Hz, J = 8.9 Hz, 1H), 6.68 (br s, 1H), 7.31 (t, J = 7.4 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.42 (d, J = 8.7 Hz, 1H), 7.61 (d, J = 7.6 Hz, 1H), 7.76 (d, J = 7.3 Hz, 2H), 8.69 (s, 1H), 9.05 (br s, 1H); MALDI-TOF MS *m*/*z*: M_n 4041, M_w 4070, M_p 4118.2 $[M + K]^+$. Calcd: M_n 4113, M_p 4160.0 $[M + K]^+$.

Biotin−PEG−NHFmoc (19). Et_3N (13 μL, 0.09 mmol) and PEG **5** (76 mg, 0.020 mmol) were added to a suspension of the biotin derivative **8** (34 mg, 0.057 mmol) in $CHCl_3$ (2 mL). The reaction was stirred at rt for 14 h, and then the excess of **8** was filtered off, and the solvent was evaporated. The crude product was dissolved in CH_2Cl_2 (1 mL), was precipitated by the addition of Et_2O (∼200 mL), and was filtered to give **19** (81 mg, 99%) as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 333 K) δ: 1.10−1.29 (m, 2H), 1.31−1.82 (m, 4H), 2.24 (t, J = 7.1 Hz, 2H), 2.43 (t, J = 6.0 Hz, 2H), 2.69 (d, J = 12.7 Hz, 1H), 2.84 (dd, J = 4.8 Hz, J = 12.7 Hz, 1H), 3.08−3.22 (m, 1H), 3.23−3.90 (m, ∼330H), 4.15 (t, J = 6.3 Hz, 1H), 4.25−4.32 (m, 1H), 4.33 (d, J = 6.7 Hz, 2H), 4.35−4.50 (m, 1H), 5.00−5.40 (m, 1H) 5.49 (br s, 1H), 5.91 (br s, 1H), 6.59 (br s, 1H), 5.96 (br s, 1H), 7.25 (t, J = 7.4 Hz, 2H), 7.32 (t, J = 7.3 Hz, 2H), 7.54 (d, J = 7.2 Hz, 2H), 7.79 (d, J = 7.3 Hz, 2H); MALDI-TOF MS *m*/*z*: M_n 4141, M_w 4176, M_p 4125.7 $[M + Na]^+$. Calcd: M_n 4096, M_p 4126.9 $[M + Na]^+$.

Man−PEG−NH$_2$ (20). Octane-1-thiol (123 μL, 0.709 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (7 μL, 0.039 mmol) were added to a solution of mannosylated PEG **16** (202 mg, 0.048 mmol) in CH_2Cl_2 (4.7 mL). The resulting solution was stirred at rt for 12 h. Then, the solvent was evaporated, and the resulting crude product was dissolved in CH_2Cl_2 (1 mL), was precipitated by the addition of Et_2O (∼200 mL), and was filtered to give **20** (186 mg 97%) as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 333 K) δ: 1.99 (s, 3H), 2.04 (s, 3H), 2.10 (s, 3H), 2.15 (s, 3H), 2.46 (t, J = 5.8, 2H), 3.10−3.20 (m, 2H), 3.34−3.99 (m, ∼330H), 4.06 (ddd, J = 2.3 Hz, J = 4.8 Hz, J = 9.3 Hz, 1H), 4.11 (dd, J = 2.3 Hz, J = 12.3 Hz, 1H), 4.29 (dd, J = 5.0 Hz, J = 12.2 Hz, 1H), 4.88 (d, J = 0.9 Hz, 1H), 5.25−5.33 (m, 2H), 5.35 (dd, J = 3.3 Hz, J = 10.0 Hz, 1H), 6.46 (br s, 1H), 7.93 (br s, 1H).

Cholesterol−PEG−NH$_2$ (21). From a solution of PEG **17** (106 mg, 0.025 mmol), octane-1-thiol (65 μL, 0.375 mmol), and DBU (4 μL, 0.025 mmol) in THF (2.5 mL), and following the same procedure as for the synthesis of **20**, the cholesterol conjugate **21** (91 mg, 90%) was obtained as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 313 K) δ: 0.70 (s, 3H), 0.86 (d, J = 1.7 Hz, 3H), 0.92 (d, J = 6.6 Hz, 3H), 1.01 (s, 3H), 1.02−1.43 (m, 14H), 1.45−1.63 (m, 6H), 1.75−2.05 (m, 6H), 2.24−2.40 (m, 2H), 2.42 (t, J = 5.9 Hz, 2H), 3.10−3.20 (m, 2H), 3.21−

3.90 (m, ~330H), 4.36−4.56 (m, 1H), 5.09 (br s, 1H), 5.34−5.38 (m, 1H), 6.57 (br s, 1H).

Coumarin−PEG−NH_2 (22). From a solution of PEG **18** (117 mg, 0.028 mmol), octane-1-thiol (72 μL, 0.426 mmol), and DBU (4 μL, 0.028 mmol) in CH_2Cl_2 (3 mL) protected from the light, and following the same procedure as for the synthesis of **20**, the coumarin conjugate **22** (108 mg, 98%) was obtained as an orange powder: ^{1}H NMR (300 MHz, $CDCl_3$, 300 K) δ: 1.23 (t, J = 7.1 Hz, 6H), 2.52 (t, J = 6.1 Hz, 2H), 3.10−3.20 (m, 2H), 3.21−3.90 (m, ~330H), 6.49 (d, J = 2.2 Hz, 1H), 6.65 (dd, J = 2.4 Hz, J = 8.9 Hz, 1H), 6.68 (br s, 1H), 7.43 (d, J = 8.9 Hz, 1H), 8.69 (s, 1H), 9.05 (br s, 1H).

Biotin−PEG−NH_2 (23). From a solution of PEG **19** (81 mg, 0.020 mmol), octane-1-thiol (50 μL, 0.28 mmol), and DBU (3 μL, 0.021 mmol) in THF (2 mL), and following the same procedure as for the synthesis of **20**, the biotin conjugate **23** (75 mg, 98%) was obtained as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 333 K) δ: 1.10−1.30 (m, 2H), 1.31−1.80 (m, 4H), 2.24 (t, J = 7.1 Hz, 2H), 2.43 (t, J = 6.0 Hz, 2H), 2.69 (d, J = 12.7 Hz, 1H) 2.84 (dd, J = 4.8 Hz, J = 12.7 Hz, 1H), 2.92−3.01 (m, 1H), 3.10−3.25 (m, 2H), 3.26−3.90 (m, ~330H), 4.25−4.35 (m, 1H), 4.40−4.55 (m, 1H), 4.60−4.80 (m, 1H), 5.00−5.40 (m, 1H) 6.20 (br s, 1H), 6.73 (br s, 1H).

Man−PEG−CO_2H (24). A solution of PEG **20** (186 mg, 0.047 mmol) and glutaric anhydride (27 mg, 0.236 mmol) in pyridine (4.7 mL) was stirred at rt for 12 h. Then, the solvent was evaporated, and the resulting crude product was dissolved in CH_2Cl_2 (10 mL). Amberlite IR-120 was added, and after 30 min of orbital stirring, the resin was filtered off. The filtrate was concentrated and then was dissolved in CH_2Cl_2 (1 mL), was precipitated by the addition of Et_2O (~200.0 mL), and was filtered to give the mannose conjugate **24** (185 mg, 97%) as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 333 K) δ: 1.94−2.00 (m, 5H), 2.04 (s, 3H), 2.10 (s, 3H), 2.15 (s, 3H), 2.28 (t, J = 7.1 Hz, 2H), 2.38 (t, J = 6.7 Hz, 2H), 2.45 (t, J = 6.0 Hz, 2H), 3.34−3.99 (m, ~330H), 4.06 (ddd, J = 2.3 Hz, J = 4.8 Hz, J = 9.3 Hz, 1H), 4.11 (dd, J = 2.3 Hz, J = 12.3 Hz, 1H), 4.29 (dd, J = 5.0 Hz, J = 12.2 Hz, 1H), 4.88 (d, J = 0.9 Hz, 1H), 5.25−5.33 (m, 2H), 5.35 (dd, J = 3.3 Hz, J = 10.0 Hz, 1H), 6.23 (br s, 1H), 6.46 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, 300 K) δ: 20.4, 20.5, 20.7, 32.9, 35.1, 36.9, 38.9, 39.0, 39.1, 62.2, 66.0, 67.0, 67.1, 68.2, 68.8, 69.3, 69.6, 69.7, 69.9, 70.1, 70.2, 70.5, 71.2, 96.5, 169.4, 169.7, 169.8, 170.4, 171.2, 172.4, 174.4; MALDI-TOF MS m/z: M_n 4138, M_w 4167, M_p 4141.7 $[M + K]^+$. Calcd: M_n 4093, M_p 40139.8 $[M + K]^+$.

Deacetylated Man−PEG−CO_2H (25). Sodium methoxide (1 M in MeOH) was added dropwise to a solution of PEG **24** (180 mg, 0.044 mmol) in dry MeOH (5.0 mL) till pH 9 was reached. Then, the reaction mixture was stirred at rt for 12 h. After neutralization with Amberlite IR-120, the mixture was filtered, and the filtrate was concentrated. The resulting crude product was dissolved in CH_2Cl_2 (1 mL), was precipitated by the addition of Et_2O (~200.0 mL), and was filtered to give the mannose conjugate **25** (160 mg, 93%) as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 333 K) δ: 1.97 (m, 2H), 2.31 (t, J = 7. 1 Hz, 2H), 2.39 (t, J = 6.7 Hz, 2H), 2.49 (t, J = 6.0 Hz, 2H), 3.34−3.99 (m, ~330H), 4.99 (d, J = 1.1 Hz, 1H), 6.70 (br s, 1H), 7.09 (br s, 1H); ^{1}H NMR (300 MHz, 300 K, D_2O) δ: 1.87 (q, J = 7.6 Hz, 1H), 2.25−2.40 (m, 4H), 2.49 (t, J = 6.1 Hz, 2H), 3.42 (dd, J = 5.2 Hz, J = 10.5 Hz, 4H), 3.45−3.99 (m, ~330H), 4.99 (d, J = 1.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, 300 K) δ: 20.8, 32.7, 35.0, 36.8, 39.1, 62.1, 62.3, 65.4, 66.5, 67.2, 67.9, 68.7, 69.6, 69.9, 70.2, 70.4, 71.3, 71.6, 71.9, 100.0, 171.6, 172.5, 174.5. MALDI-TOF MS m/z: M_n 4033, M_w 4057, M_p 4041.4 $[M + Na]^+$. Calcd: M_n 3925, M_p 3955.6 $[M + Na]^+$.

Cholesterol−PEG−CO_2H (26). From a solution of PEG **21** (91 mg 0.022 mmol) and glutaric anhydride (12 mg, 0.105 mmol) in pyridine (2.2 mL), and following the same procedure as for the synthesis of **24**, the cholesterol conjugate **26** (87 mg, 93%) was obtained as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 333 K) δ: 0.70 (s, 3H), 0.86 (d, J = 1.7 Hz, 3H), 0.88 (d, J = 1.7 Hz, 3H), 0.92 (d, J = 6.6 Hz, 3H), 1.01 (s, 3H), 1.02−1.43 (m, 14H), 1.45−1.63 (m, 6H), 1.75−2.05 (m, 8H), 2.24−2.41 (m, 6H), 2.44 (t, J = 5.9 Hz, 2H), 3.20−3.90 (m, ~330H), 4.43−4.56 (m, 1H), 5.09 (br s, 1H), 5.34−5.38 (m, 1H), 6.57 (br s, 1H); ^{1}H NMR (300 MHz, D_2O, 300 K) δ: 0.60−1.00 (m, 15H), 1.10−1.75 (m, 20H), 1.82−2.13 (m, 8H), 2.13−2.37 (m, 6H) 2.38−2.58 (m, 2H), 3.20−3.90 (m, ~330H), 4.16−4.25 (m, 1H), 5.38−5.42 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$, 300 K) δ: 11.7, 18.6, 19.2, 20.7, 21.0, 22.4, 22.7, 23.7, 24.1, 27.9, 28.0, 28.1, 29.5, 31.8, 31.9, 32.7, 35.0, 35.7, 35.9, 36.4, 36.9, 38.5, 39.0, 39.1, 39.4, 39.6, 40.6, 42.2, 49.9, 53.4, 56.0, 56.6, 67.2, 69.7, 69.8, 70.0, 70.1, 70.2, 70.3, 70.5, 74.3, 122.3, 139.8, 140.2, 143.9, 156.1, 171.2, 172.5, 174.6; MALDI-TOF MS m/z: M_n 4258, M_w 4295, M_p 4250.2 $[M + Na]^+$. Calcd: M_n 4174, M_p 4205.1 $[M + Na]^+$.

Coumarin−PEG−CO_2H (27). From a solution of PEG **22** (101 mg, 0.026 mmol) and glutaric anhydride (15 mg, 0.131 mmol) in pyridine (3 mL) protected from the light, and following the same procedure as for the synthesis of **24**, the coumarin conjugate **27** (98 mg, 94%) was obtained as an orange powder: ^{1}H NMR (400 MHz, $CDCl_3$, 300 K) δ: 1.17 (t, J = 7.1 Hz, 6H), 1.91−2.03 (m, 2H), 2.23 (t, J = 7.2 Hz, 2H), 2.31 (t, J = 6.8 Hz, 2H), 2.45 (t, J = 6.1 Hz, 2H), 3.33−3.91 (m, ~330H), 6.42 (d, J = 2.3 Hz, 1H), 6.58 (dd, J = 2.4 Hz, J = 9.0 Hz, 1H), 6.82 (br s, 1H), 7.37 (d, J = 9.0 Hz, 1H), 8.69 (s, 1H), 8.98 (br s, 1H); ^{1}H NMR (300 MHz, D_2O, 300 K) δ: 1.22 (t, J = 7.1 Hz, 6H), 1.75−1.95 (m, 2H), 2.18 (t, J = 7.4 Hz, 2H), 2.25 (t, J = 6.9 Hz, 2H), 2.45 (t, J = 6.0 Hz, 2H), 3.20−3.90 (m, ~330H), 6.67−6.71 (m, 1H), 6.88−6.96 (m, 1H), 7.63 (d, J = 9.0 Hz 1H), 8.63 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, 300 K) δ: 12.4, 20.8, 32.7, 35.0, 36.8, 39.1, 39.2, 39.3, 45.0, 69.9, 70.0, 70.2, 70.4, 70,5, 96.5, 108.3, 109.9, 110.2, 131.0, 148.0, 152.5, 157.6, 162.6, 163.3, 171.3, 172.5, 174.4; MALDI-TOF MS m/z: M_n 3950, M_w 3974, M_p 4007.0 $[M + K]^+$. Calcd: M_n 4005, M_p 4050.7 $[M + K]^+$.

Biotin−PEG−CO_2H (28). From a solution of PEG **23** (71 mg, 0.018 mmol) and glutaric anhydride (10 mg, 0.088 mmol) in pyridine (2 mL), and following the same procedure as for the synthesis of **24**, the biotin conjugate **28** (65 mg, 89%) was obtained as a white powder: ^{1}H NMR (400 MHz, $CDCl_3$, 333 K) δ: 1.10−1.30 (m, 2H), 1.31−1.80 (m, 4H), 1.95 (q, J = 6.9 Hz, 2H), 2.15 (t, J = 6.7 Hz, 2H), 2.22 (t, J = 7.1 Hz, 2H), 2.32 (t, J = 6.8 Hz, 2H), 2.41 (t, J = 5.5 Hz, 2H), 2.75 (d, J = 12.8 Hz, 1H), 2.93 (dd, J = 4.9 Hz, J = 12.8 Hz, 1H), 3.00−3.20 (m, 1H), 3.25−3.90 (m, ~330H), 4.25−4.32 (m, 1H), 4.35−4.50 (m, 1H), 4.60−4.80 (m, 1H), 5.00−5.40 (m, 1H), 6.20 (br s, 1H), 6.70 (br s, 1H); ^{1}H NMR (300 MHz, D_2O, 300 K) δ: 1.10−1.30 (m, 2H), 1.31−1.49 (m, 1H), 1.50−1.75 (m, 2H), 1.75−1.95 (m, 1H), 2.10−2.35 (m, 6H), 2.55 (t, J = 6.0 Hz, 2H), 2.75 (d, J = 13.3 Hz, 1H), 2.93 (dd, J = 4.9 Hz, J = 12.8 Hz, 1H), 3.00−3.20 (m, 1H), 3.25−3.90 (m, ~330H), 4.38−4.45 (m, 1H), 4.55−4.65 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, 300 K) δ: 20.7, 25.3, 27.9, 32.8, 35.1, 35.7, 36.7, 39.2, 40.5, 55.2, 60.1, 61.8, 67.2, 69.7, 69.8, 70.0, 70.1, 70.5, 171.8, 172.5, 173.2, 174.4; MALDI-TOF MS m/z: M_n 4030, M_w 4060, M_p 4035.3 $[M + K]^+$. Calcd: M_n 3988, M_p 4034.9 $[M + K]^+$.

Chitosan-*g*-PEG−Man 29A. Chitosan·HCl (31.6 mg, 0.159 mmol), PEG **25** (3.8 mg, 0.97 μmol), and NHS (0.5 mg, 4.34 μmol, 100 μL of a 5 mg/mL solution) were dissolved in H_2O (4.5 mL). Then, EDC·HCl (9.2 mg, 0.048 mmol) was added in four portions every 30 min. The resulting solution was stirred at rt for 22 h and then was ultrafiltered (YM30) with H_2O and was lyophilized to afford **29A** (33.9 mg, DS 0.5%, 83% yield, 97% mass recovery) as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 3.10−3.30 (m, 84H), 3.31−4.30 (m, 671H), 4.55−4.70 (m), 4.80−4.95 (m).

Chitosan-*g*-PEG−Man 29B. From a solution of chitosan·HCl (25.0 mg, 0.126 mmol), PEG **25** (14.4 mg, 3.67 μmol), NHS (0.84 mg, 7.29 μmol, 100 μL of a 8.4 mg/mL solution), and EDC·HCl (35.0 mg, 0.183 mmol) in H_2O (3.6 mL), and following the same procedure as above, **29B** (34.0 mg, DS 2.1%, 73% yield, 96% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 3.10−3.30 (m, 84H), 3.31−4.30 (m, 1206H), 4.55−4.70 (m), 4.80−4.95 (m).

Chitosan-*g*-PEG−Man 29C. From a solution of chitosan·HCl (25.0 mg, 0.126 mmol), PEG **25** (28.8 mg, 7.34 μmol), NHS (1.68 mg, 14.59 μmol, 100 μL of a 16.8 mg/mL solution), and EDC·HCl (35.0 mg, 0.183 mmol) in H_2O (3.6 mL), and following the same procedure as above, **29C** (43.0 mg, DS 4.2%, 72% yield, 94% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.45 (t, J = 7.3 Hz), 2.55 (t, J = 6.1 Hz), 3.10−3.30 (m, 84H), 3.31−4.30 (m, 1911H), 4.55−4.70 (m), 4.80−4.95 (m).

Chitosan-*g*-PEG−Cholesterol 30A. From a solution of chitosan·HCl (36.2 mg, 0.183 mmol), PEG **26** (4.6 mg, 1.10 μmol,), NHS (0.63 mg, 5.47 μmol, 100 μL of a 6.3 mg/mL solution), and EDC·HCl (10.5 mg, 0.055 mmol) in H_2O (5.1 mL), and following the same procedure as above, **30A** (39.4 mg, DS 0.5%, 83% yield, 100% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 0.71−1.02 (m), 1.03−1.61 (m), 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 3.10−3.30 (m, 85H), 3.31−4.30 (m, 708H), 4.55−4.70 (m) 4.80−4.95 (m).

Chitosan-*g*-PEG−Cholesterol 30B. From a solution of chitosan·HCl (27.4 mg, 0.138 mmol), PEG **26** (16.7 mg, 4.00 μmol), NHS (0.92 mg, 7.99 μmol, 100 μL of a 9.2 mg/mL solution), and EDC·HCl (38.4 mg, 0.200 mmol) in H_2O (3.8 mL), and following the same procedure as above, **30B** (41.9 mg, DS 2.7%, 93% yield, 96% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 0.70−1.00 (m), 1.01−1.61 (m), 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 3.10−3.30 (m, 84H), 3.31−4.30 (m, 1399H), 4.55−4.70 (m), 4.80−4.95 (m).

Chitosan-*g*-PEG−Cholesterol 30C. From a solution of chitosan·HCl (25.0 mg, 0.126 mmol), PEG **26** (31.0 mg, 7.43 μmol), NHS (1.7 mg, 14.77 μmol, 100 μL of a 17 mg/mL solution), and EDC·HCl (35.0 mg, 0.183 mmol) in H_2O (3.6 mL), and following the same procedure as above, **30C** (52.0 mg, DS 5.4%, 92% yield, 96% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 0.70−1.00 (m) 1.01−1.61 (m), 1.80−1.93 (m), 2.00−2.20 (m, 41.1 H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 3.10−3.30 (m, 84H), 3.31−4.30 (m, 2305H), 4.55−4.70 (m), 4.80−4.95 (m).

Chitosan-*g*-PEG−Coumarin 31A. From a solution of chitosan·HCl (25.0 mg, 0.126 mmol), PEG **27** (3.0 mg, 0.75 μmol), NHS (0.43 mg, 3.74 μmol, 100 μL of a 4.3 mg/mL solution), and EDC·HCl (7.3 mg, 0.038 mmol) in H_2O (3.6 mL) protected from the light, and following the same procedure as above, **31A** (27.0 mg, DS 0.5%, 83% yield, 98% mass recovery) was obtained as a yellow foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.10−1.20 (m), 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 3.10−3.30 (m, 85H), 3.31−4.30 (m, 669H), 4.55−4.70 (m), 4.80−4.95 (m).

Chitosan-*g*-PEG−Coumarin 31B. From a solution of chitosan·HCl (25.0 mg, 0.126 mmol), PEG **27** (14.7 mg, 3.67 μmol), NHS (0.84 mg, 7.29 μmol, 100 μL of a 8.4 mg/mL solution), and EDC·HCl (35.0 mg, 0.183 mmol) in H_2O (3.6 mL) protected from the light, and following the same procedure as above, **31B** (36.0 mg, DS 2.2%, 77% yield, 100% mass recovery) was obtained as a yellow foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.13 (t, J = 7.2 Hz), 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 3.10−3.30 (m, 84H), 3.31−4.30 (m, 1386H), 4.55−4.70 (m), 4.80−4.95 (m), 7.63 (t, J = 8.7 Hz), 8.07−8.17 (m), 8.88 (s), 8.95 (s).

Chitosan-*g*-PEG−Coumarin 31C. From a solution of chitosan·HCl (25.0 mg, 0.126 mmol), PEG **27** (29.7 mg, 7.42 μmol), NHS (1.7 mg, 14.77 μmol, 100 μL of a 17 mg/mL solution), and EDC·HCl (35.0 mg, 0.183 mmol) in H_2O (3.6 mL) protected from the light, and following the same procedure as above, **31C** (46.2 mg, DS 4.4%, 75% yield, 98% mass recovery) was obtained as a yellow foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.13 (t, J = 7.2 Hz), 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 3.10−3.30 (m, 82H), 3.31−4.30 (m, 1968H), 4.55−4.70 (m), 4.80−4.95 (m), 7.63 (t, J = 8.7 Hz), 8.07−8.17 (m), 8.88 (s), 8.95 (s).

Chitosan-*g*-PEG−Biotin 32A. From a solution of chitosan·HCl (36.7 mg, 0.185 mmol), PEG **28** (4.5 mg, 1.13 μmol), NHS (0.64 mg, 5.56 μmol, 100 μL of a 6.4 mg/mL solution), and EDC·HCl (10.5 mg, 0.055 mmol) in H_2O (5.3 mL), and following the same procedure as above, **32A** (39.2 mg, DS 0.5%, 83% yield, 98% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.13−1.36 (m), 2.00−2.20 (m, 41.1H), 3.10−3.30 (m, 86H), 3.31−4.30 (m, 683H), 4.55−4.70 (m), 4.80−4.95 (m).

Chitosan-*g*-PEG−Biotin 32B. From a solution of chitosan·HCl (25.6 mg, 0.129 mmol), PEG **28** (15.0 mg, 3.76 μmol), NHS (0.9 mg, 7.82 μmol, 100 μL of a 9.0 mg/mL solution), and EDC·HCl (36.0 mg, 0.188 mmol) in H_2O (3.6 mL), and following the same procedure as above, **32B** (35.8 mg, DS 2.0%, 79% yield, 100% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.13−1.36 (m), 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 2.77 (d, J = 13.7 Hz), 3.10−3.30 (m, 84H), 3.31−4.30 (m, 1176H), 4.35−4.52 (m), 4.55−4.70 (m), 4.80−4.95 (m).

Chitosan-*g*-PEG−Biotin 32C. From a solution of chitosan·HCl (25.5 mg, 0.129 mmol), PEG **28** (30.4 mg, 7.62 μmol,), NHS (1.7 mg, 14.77 μmol, 100 μL of a 17 mg/mL solution), and EDC·HCl (36.0 mg, 0.18 mmol) in H_2O (3.6 mL), and following the same procedure as above, **32C** (45.9 mg, DS 4.6%, 78% yield, 94% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.13−1.36 (m), 1.37−1.79 (m), 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 2.77 (d, J = 13.7 Hz), 3.10−3.30 (m, 82H), 3.31−4.30 (m, 2018H), 4.35−4.52 (m), 4.55−4.70 (m), 4.80−4.95 (m).

Multifunctional Chitosan-*g*-PEG 33. From a solution of chitosan·HCl (15.5 mg, 7.84 μmol), **25** (3.0 mg, 0.76 μmol), **27** (3.0 mg, 0.75 μmol), **28** (3.0 mg, 0.75 μmol), NHS (0.52 mg, 4.52 μmol), and EDC·HCl (21.8 mg, 0.114 mmol) in H_2O (3.6 mL), and following the same procedure as above, **33** (19.1 mg, DS 0.67% for each PEG, 70% yield, 88% mass recovery) was obtained as a yellow foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 1.13−1.35 (m), 1.80−1.93 (m), 2.00−2.20 (m, 41.1H), 2.23−2.25 (m), 2.40−2.47 (m), 2.50−2.60 (m), 3.10−3.30 (m, 82H), 3.30−4.30 (m, 1156H), 4.55−4.70 (m), 4.80−4.95 (m).

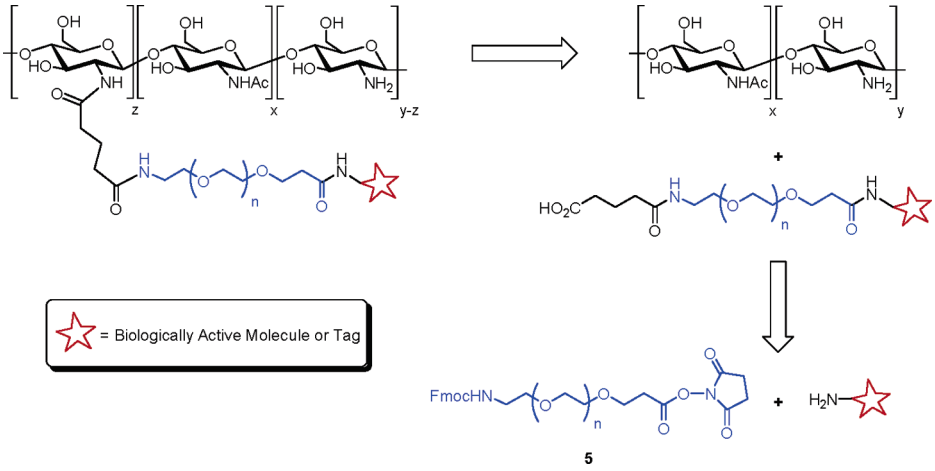
Multifunctional Chitosan-*g*-PEG 35. From a solution of chitosan·HCl (25.0 mg, 0.126 mmol), **34** (29.0 mg, 5.67 μmol) (*23*), **25** (2.5 mg, 0.64 μmol), NHS (1.45 mg, 12.60 μmol), and EDC·HCl (30.2 mg, 0.157 mmol) in H_2O (3.6 mL), and following the same procedure as above, **35** (52.0 mg, DS 4.12% for MeO−PEG and 0.46% for mannose−PEG, 78% yield, 97% mass recovery) was obtained as a white foam: ^{1}H NMR (400 MHz, 2% DCl in D_2O, 300 K) δ: 2.00−2.20 (m, 41.1H), 3.10−3.30 (m, 82H), 3.30−4.30 (m, 2560H), 4.55−4.70 (m), 4.80−4.95 (m).

Results and Discussion

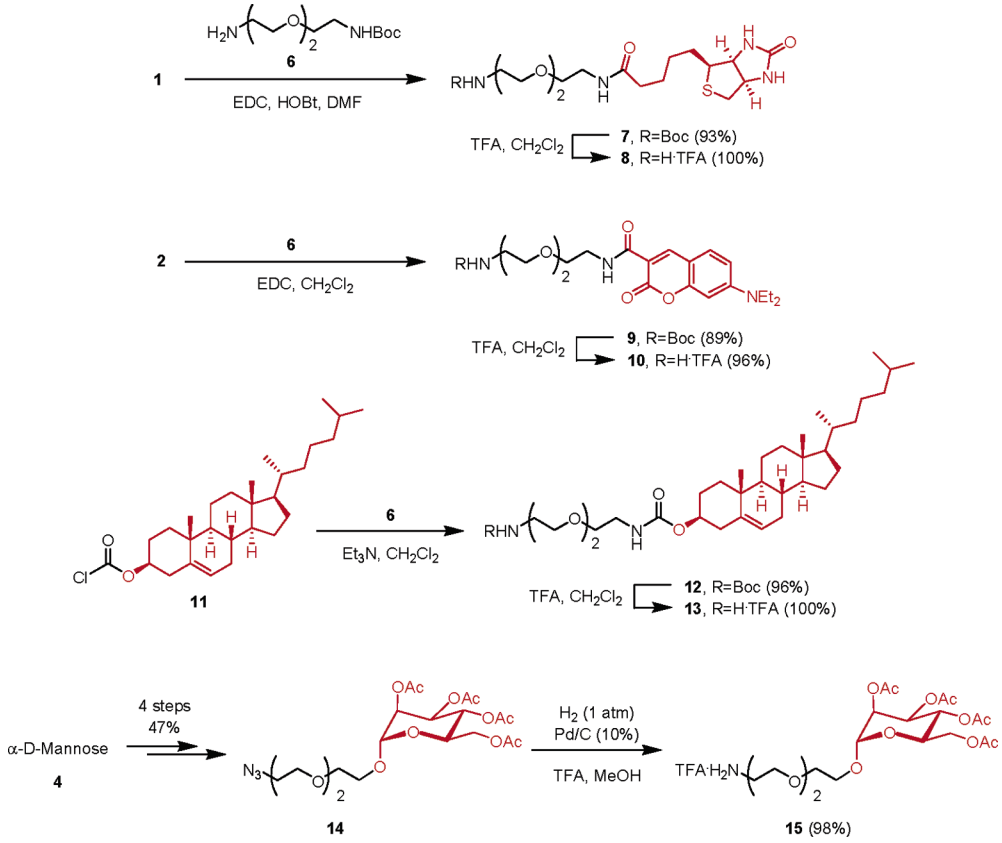
The chitosan-*g*-PEG functionalized with the active molecules/tags **1**−**4** at the distal end of the PEG chains has been synthesized according to the retrosynthetic analysis depicted in Scheme 1. This implies the use of an amide bond between the amino groups of chitosan and a carboxylic acid functionalized PEG. This grafting linkage was selected because of the high yield and reproducibility of the process.[7] With the purpose of locating the active molecules/tags **1**−**4** at the opposite end of the PEG chains, a heterodifunctional PEG such as **5**, with a protected (Fmoc) amino group at one end and a *N*-hydroxysuccinimide (NHS) active ester at the other, was required. In this way, conjugation of the active ester of **5** with amino-functionalized active molecules/tags, followed by deprotection of the Fmoc group and reaction with glutaric anhydride, produced the required PEG incorporating the active molecules/tags at one end and a carboxylic acid amenable for grafting to chitosan at the other.

Although PEG technology has amply developed in the last years, reactions on chain ends are frequently not conveniently

Scheme 1



Scheme 2

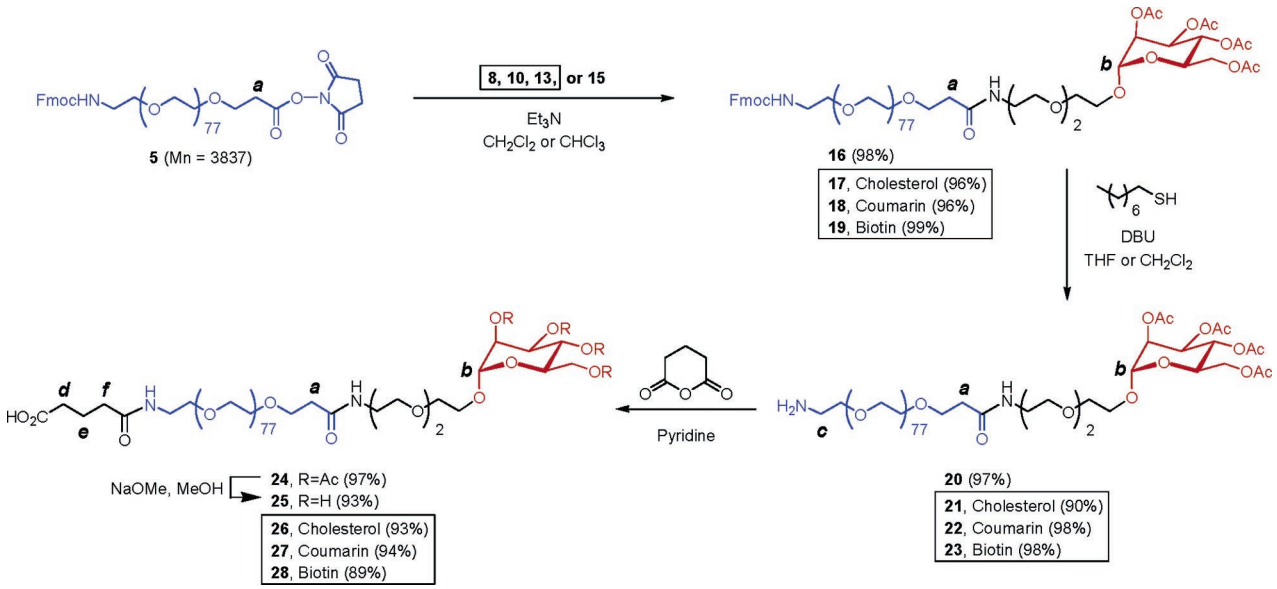


monitored and quantified, leading to PEG conjugates that suffer from poor characterization, purity, and reproducibility of their syntheses. In our case, to ensure the purity of the PEG end groups, and to avoid complex polymeric mixtures, we present reaction conditions that proceed quantitatively for each synthetic step in the functionalization of PEG as well as complete characterization of the PEG conjugates (^{1}H and ^{13}C NMR and MALDI-TOF MS) and graft copolymers (^{1}H NMR).

With the aim of rendering the active molecules/tags amenable for conjugation to the active ester of PEG **5**, and also to improve their solubility, **1**−**4** were first derivatized with a monoprotected tris(ethylene glycol)-diamine linker **6**[3] (Scheme 2). Thus, amide coupling between biotin **1** and **6** (EDC, HOBt, DMF) led to the protected biotin derivative **7** in 93% yield. Deprotection of the Boc group of **7** with TFA in CH_2Cl_2 afforded the amino-functionalized biotin **8** quantitatively. In the same way, coumarin **2** was reacted with linker **6** (EDC, CH_2Cl_2) to give amide **9** (89%) that after Boc deprotection (TFA, CH_2Cl_2) led to the amino-derived coumarin **10** in 96% yield. Likewise, treatment of the cholesterol chloroformate **11** with **6** (Et_3N, CH_2Cl_2) afforded carbamate **12** in excellent yield (96%) that after deprotection gave the amino cholesterol derivative **13** quantitatively. For the synthesis of the amino-functionalized mannoside **15**, α-D-mannose (**4**) was first converted into the azido-mannoside **14** (47% overall yield)[26] and then was hydrogenated under acidic conditions (98%).

Once the amino-functionalized active molecules/tags **8**, **10**, **13**, and **15** were in hand, their conjugation with a narrow polydispersity heterodifunctional PEG **5** (M_n = 3837, M_w = 3890 by MALDI-TOF) was easily carried out (Scheme 3). The resulting conjugates were purified by precipitation from diethyl ether−dichloromethane (Et_2O−CH_2Cl_2). The properties of PEG

Scheme 3



as a soluble polymeric support render unnecessary solvent extractions and tedious chromatographic purifications.[27] Thus, reaction of PEG **5** with 3.1 equiv of the peracetylated mannoside **15** (Et_3N, CH_2Cl_2) afforded the PEG−mannose conjugate **16** in an excellent yield (98%) after precipitation ($Et_2O-CH_2Cl_2$). The incorporation of the mannose residue in **16** was established by ^1H NMR ($CDCl_3$) because of the presence of four singlets at 1.99, 2.04, 2.10, and 2.15 ppm corresponding to the acetyl-protecting groups and a doublet at 4.88 ppm corresponding to the anomeric proton (α configuration as indicated by the coupling constant, $J = 0.9$ Hz) (Figure 4). Then, deprotection

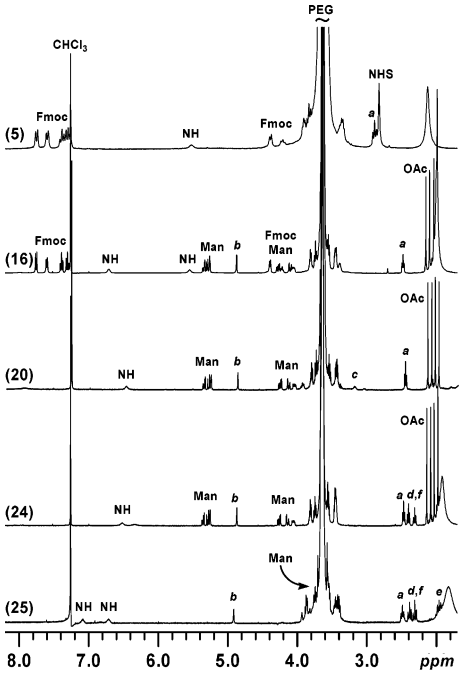


Figure 4. ^1H NMR spectra of starting PEG **5** and mannose conjugates **16**, **20**, **24**, and **25** (400 MHz, $CDCl_3$).

of the Fmoc group in **16** was effectively performed with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (octane-1-thiol, CH_2Cl_2)[28] leading to the PEG amino **20** in an excellent 97% yield after precipitation ($Et_2O-CH_2Cl_2$). The completeness of the deprotection of the Fmoc was easily monitored by ^1H NMR ($CDCl_3$) thanks to the disappearance of its characteristic aromatic signals between 7.31 and 7.76 ppm. Conversion of the primary amino group of **20** into the carboxylic acid functionality required for the grafting to chitosan was realized by treatment with 5 equiv of glutaric anhydride in pyridine. The desired conjugate **24** was obtained in 97% yield, after treating the concentrated reaction mixture with Amberlite IR-120 and subsequent precipitation ($Et_2O-CH_2Cl_2$). The incorporation of the glutaric acid linker was unambiguously established by the presence of two triplets at 2.28 ($J = 7.1$ Hz) and 2.38 ppm ($J = 6.7$ Hz) and one multiplet between 1.95 and 1.98 ppm. Finally, deprotection of the acetyl groups with sodium methoxide (NaOMe) in MeOH afforded the desired mannosylated PEG **25** in an excellent 93% yield after neutralization with Amberlite IR-120 and precipitation ($Et_2O-CH_2Cl_2$). The complete deprotection of the acetyl groups in **25** was demonstrated by the disappearance of their characteristic ^1H NMR signals, while the α configuration at the mannose residue remained untouched as indicated by the presence of a doublet at 4.99 ppm with a coupling constant $J = 1.1$ Hz.

PEG conjugates **16**, **24**, and **25** were also characterized by MALDI-TOF spectrometry (Figure 5 and Table 1). The spectrum of **16** revealed a series of 44 Da spaced peaks corresponding to the potassium adducts, accompanied by lower intensity peaks from the sodium adducts. No signals resulting from unreacted **5** or side products were seen confirming the purity of the product. Also, experimental M_n and M_p were in agreement with calculated values. Similarly, the spectra of **24** and **25** revealed pure conjugates as the sole products with well-resolved potassium and sodium adducts. Again, the perfect match between experimental and calculated M_n and M_p values confirmed the identity of the conjugates and the purity of the end groups.

Likewise, when the biotin, coumarin, and cholesterol derivatives **8**, **10**, and **13** (Scheme 3) were subjected to the same synthetic transformations (conjugation to the PEG **5**, deprotection of the Fmoc group, and reaction with glutaric anhydride), the corresponding PEG−CO_2H conjugates incorporating cholesterol (**26**, 82%), the coumarin tag **2** (**27**, 88%), and biotin (**28**, 77%) were obtained in excellent overall yields. As in the case of the mannose derivatives, the completion of the reactions,

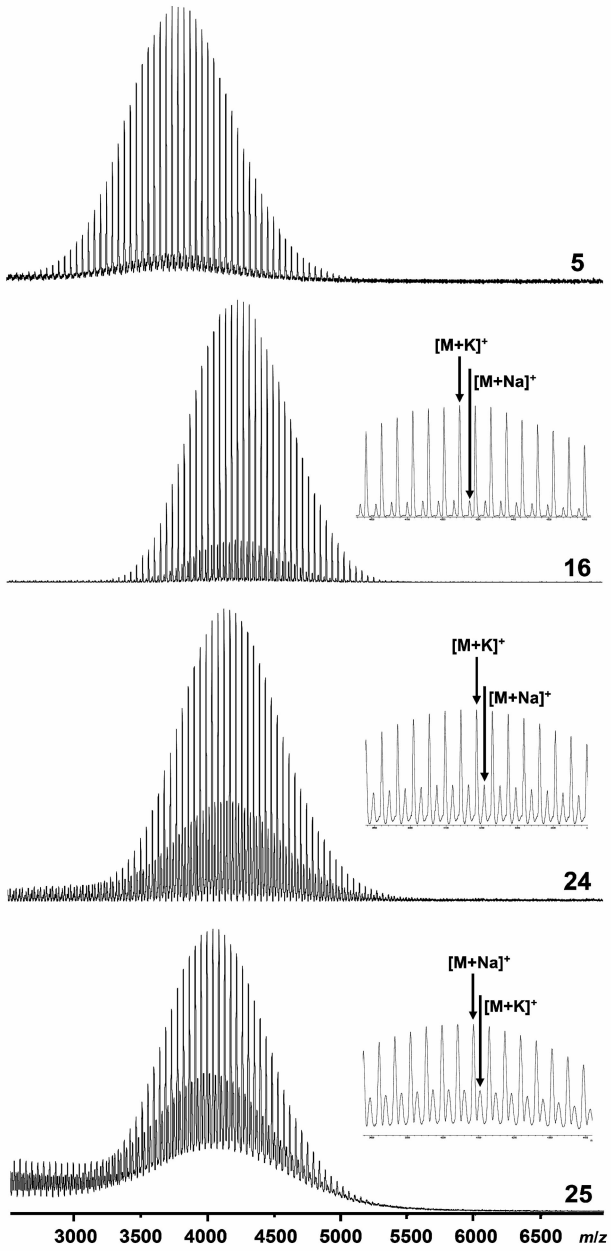


Figure 5. MALDI-TOF spectra of starting PEG **5** and mannose conjugates **16**, **24**, and **25**.

Table 1. Molecular Weight of PEG Conjugates Determined by MALDI-TOF MS

PEG	M_n[a]		M_w	M_p[a]		PDI
5	3837		3890	3866.5	[3867.5][b]	1.009
16	4269	[4207]	4293	4247.7	[4248.0][c]	1.005
17	4359	[4282]	4385	4417.5	[4328.4][c]	1.005
18	4041	[4113]	4070	4118.2	[4160.0][c]	1.010
19	4141	[4096]	4176	4125.7	[4126.9][b]	1.008
24	4138	[4093]	4167	4141.7	[4139.8][c]	1.006
25	4034	[3925]	4057	4041.4	[3955.6][b]	1.005
26	4258	[4174]	4295	4250.2	[4205.1][b]	1.009
27	3950	[4005]	4034	4007.0	[4050.7][c]	1.010
28	4030	[3988]	4060	4035.3	[4034.9][c]	1.005

[a] Numbers in brackets are calculated molecular weights. [b] $[M + Na]^+$. [c] $[M + K]^+$.

and the purity of the products, was unambiguously established by ^1H and ^{13}C NMR and MALDI-TOF MS (Table 1).

The grafting of the functionalized PEG−CO_2H (**25**−**28**) to chitosan [DA 14% by ^1H NMR,[22] M_w 8.0 10^4 g/mol by SEC-MALLS][24,25] was conveniently realized in H_2O at pH 4.6−4.9

Table 2. DS and Yields of Grafting of the Functionalized PEG-Grafted Chitosans Obtained under Conditions A−C

PEG (**25**−**28**)	DS (yield) cond. A[a]	DS (yield) cond. B[b]	DS (yield) cond. C[c]
mannose (**29**)	0.5 (83%)	2.1 (73%)	4.2 (72%)
cholesterol (**30**)	0.5 (83%)	2.7 (93%)	5.4 (92%)
coumarin (**31**)	0.5 (83%)	2.2 (77%)	4.4 (75%)
biotin (**32**)	0.5 (83%)	2.0 (70%)	4.6 (78%)

[a] Conditions A: PEG (0.60 mol %), EDC (30 mol %), NHS (3 mol %). [b] Conditions B: PEG (2.90 mol %), EDC (145 mol %), NHS (5.80 mol %). [c] Conditions C: PEG (5.87 mol %), EDC (147 mol %), NHS (11 mol %). The mol % refers to 100 mol of glucosamine/*N*-acetylglucosamine repeating units.

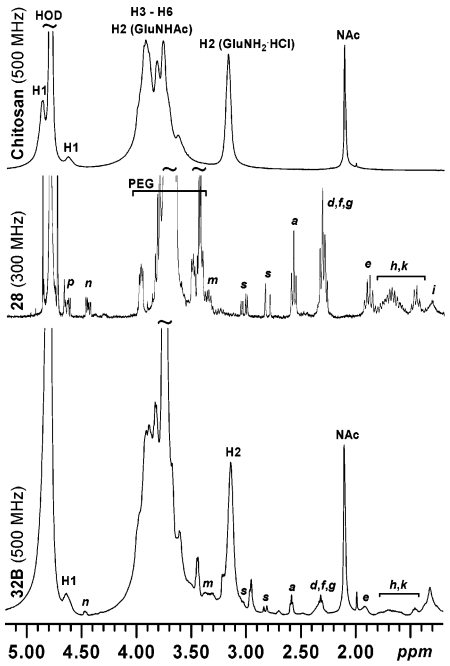
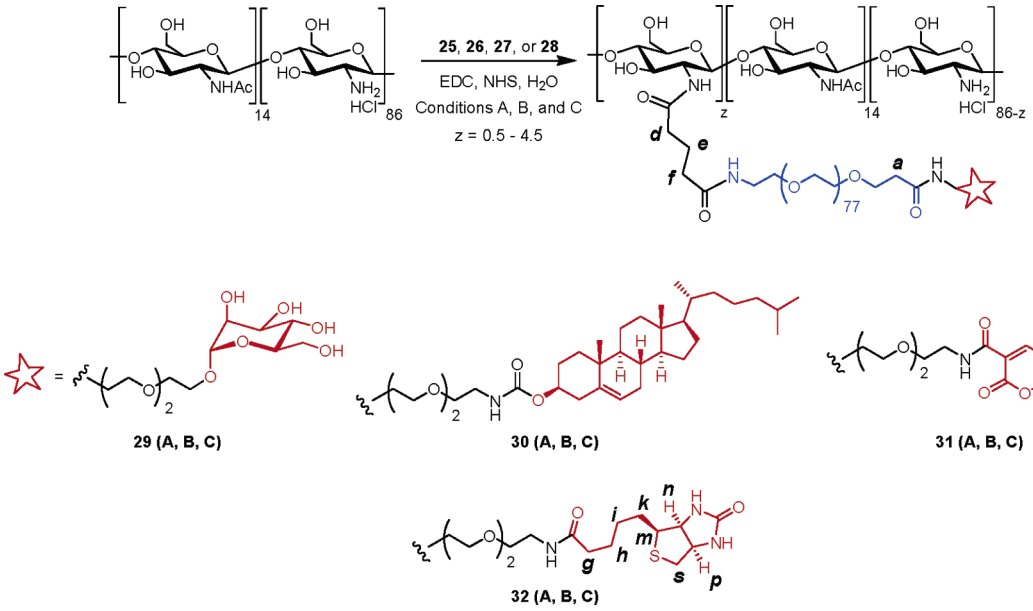


Figure 6. ^1H NMR spectra of starting chitosan, biotin−PEG−CO_2H conjugate **28**, and chitosan−PEG−biotin graft copolymer **32B** (D_2O).

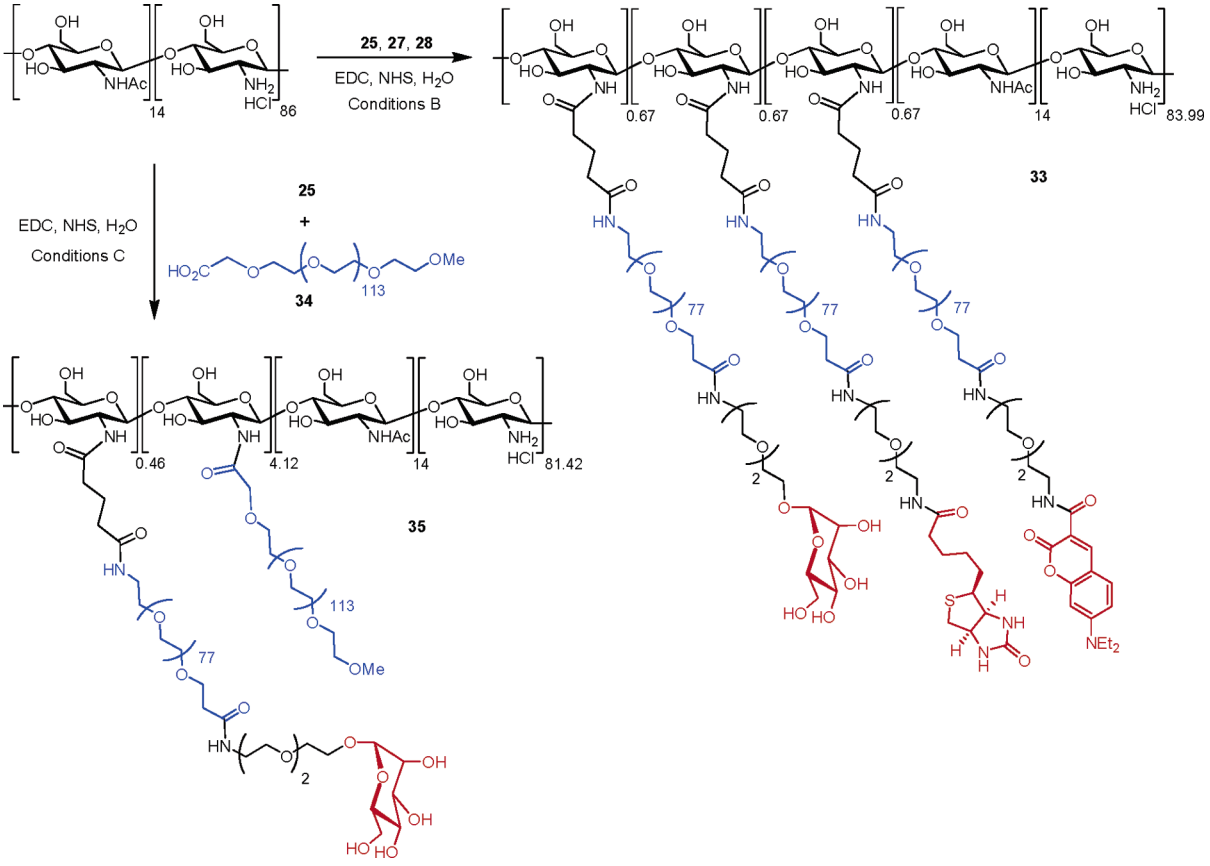
with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Degrees of substitution of PEG (DS) in the range 0.5−4.5% were pursued in line with previous results regarding the biocompatibility and stability of chitosan-*g*-PEG nanostructures.[7,13,14] With this aim, experimental conditions using different molar ratios between repeating units of chitosan and PEG [100:0.60 (conditions A), 100:2.90 (conditions B), and 100:5.87 (conditions C); Table 2] were used. The resulting PEG-grafted chitosans (mannose [**29** (**A**, **B**, **C**)], cholesterol [**30** (**A**, **B**, (C)], coumarin [**31** (**A**, **B**, **C**)], and biotin [**32** (**A**, **B**, **C**)]) were obtained with very good to excellent yields of grafting (70−93%) and showed DS around 0.5, 2.1, and 4.5%, respectively for conditions A, B, and C. (Scheme 4). Purification of the reaction mixtures was realized by means of ultrafiltration through Amicon YM30 membranes, a technique that in our hands allowed an effective separation of the graft copolymers from the reagents in excess and urea and delivered pure products with excellent mass recoveries (typically 94−100%) in considerably less time than standard size exclusion chromatography.[10]

The incorporation of the PEG chains in the resulting graft copolymers was established by ^1H NMR (D_2O) because of the appearance of a sharp singlet at 3.70 ppm corresponding to the PEG methylene protons. In the case of the copolymers with higher DS (those obtained under conditions B and C), signals of lower intensity corresponding to the active molecules/tags

Scheme 4



Scheme 5



were also clearly recognized, as shown in Figure 6 for **32B**. Also, in the series of the coumarin conjugates **31**, the grafting of the PEG **27** could be undoubtedly established by fluorescence thanks to the appearance of a maximun of emission at 476 nm, characteristic of dye **2**.

Multifunctional nanostructures incorporating various active molecules/tags on their surface are becoming very popular because of the unquestionable benefit of having ligands, targeting molecules, and dyes, for example, together on a single particle.[29] Encouraged by the success and reproducibility of the above grafting of functionalized PEG onto chitosan, the possibility of obtaining multifunctional chitosan-*g*-PEG was investigated. For the sake of synthetic efficiency, the incorporation of the different active molecules/tags should be ideally performed in a single operation. Also, since the various functionalized PEG−CO_2H tested has shown similar reactivity against chitosan, it could be anticipated that the ratio among PEGylated active molecules/tags in the multifunctional graft copolymers should be easily controlled by their feed ratio in the reaction. To test the feasibility of such an approach, equimolecular amounts of PEG conjugates **25**, **27**, and **28** were reacted with chitosan under conditions B (Scheme 5). The resulting multi-

functional graft copolymer (**33**) incorporating mannose, the coumarin dye **2**, and biotin was obtained with a 70% yield of grafting, corresponding to a DS of 0.67% for each of the active molecules/tags. Copolymers like **33** are envisioned as attractive materials with interesting applications in drug delivery and antiadhesive therapy because of their multifunctional nature.

Similarly, the use of the above technology allows the simultaneous grafting of both functionalized and unfunctionalized PEG to chitosan. For example, when mannosylated PEG **25** and the methoxy-PEG (**34**) (molar ratio **25**:**34**, 1:9) were reacted with chitosan under conditions C, block copolymer **35** was obtained with a 78% grafting (DS 0.46 and 4.12% for the mannosylated and methoxy caped PEG, respectively).

Conclusions

Four different PEG−CO_2H conjugates (**25**−**28**) incorporating mannose, cholesterol, the coumarin dye **2**, and biotin have been synthesized and completely characterized by NMR and MALDI-TOF. Conjugation of **25**−**28** to chitosan under aqueous conditions (EDC, NHS) led to the corresponding graft copolymers **29**−**32** in very good to excellent yields and nearly quantitative mass recoveries after purification by ultrafiltration. By adjusting the molar ratio between PEG and chitosan (conditions A−C), graft copolymers with DS between 0.5 and 4.5% were routinely obtained. Characterization of these graft copolymers has been performed by ^{1}H NMR. Interestingly, when under similar reaction conditions and mixtures of PEG differently functionalized were grafted to chitosan, multifunctional PEG-grafted chitosans were obtained. The usefulness of these materials in active targeting and antiadhesive therapy has been recently demonstrated by the employment of the copolymer **32A**, functionalized with biotin, in the development of immunonanoparticles as promising drug carriers across the blood−brain barrier.[30]

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