

SUPPORTING INFORMATION

pH-Responsive Glycopolymer Nanoparticles as Potential Candidates for Targeted Delivery of Anticancer Drugs

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1. EXPERIMENTAL

1.1. Materials

Methacrylic acid (MAA, 99%, contains 250 ppm mono methyl ether hydroquinone (MEHQ) as inhibitor), poly(ethylene glycol) methyl ether methacrylate (OEGMA, average $M_n \approx 300$ g.mol⁻¹, contains 100 ppm MEHQ and 300 ppm butylated hydroxytoluene (BHT) as inhibitor), 2-Cyano-2-propyl benzodithioate (CPBDT, 97%), sodium borohydride (NaBH₄, 98%, granular, 10-40 mesh) and gold nanoparticle suspension (40 nm) that stabilized with citrate were purchased from Sigma Aldrich Chemical Company. MAA and OEGMA was passed through a short column of basic alumina in order to remove MEHQ inhibitor prior to polymerization. 4,4'-azobis-(2-methylpropionitrile) (AIBN) and H₂SO₄-silica catalyst were previously synthesized within the group. All other reagents and solvents were obtained at the highest purity available from Sigma Aldrich Chemical Company and used as received unless stated otherwise. Water (H₂O, HiPerSolv Chromanorm for HPLC from VWR International) was used throughout the study. Dialysis tubes were purchased from Spectrum Laboratories. Copper coated 3.05mm diameter square carbon film mesh grids were purchased from Agar Scientific. Doxorubicin (DOX), Cysteine (Cys), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich. Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin (10,000 UI/mL), L-Glutamine (200 mM), trypsin/EDTA (0.05% trypsin; 0.20 g/L EDTA), and phosphate buffered saline (PBS) used in cell culture experiments were purchased from Lonza. Fetal bovine serum (FBS) was obtained from Biowest. 1,1'-carbonyldiimidazole (CDI), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), hydrochloric acid (HCl), and sodium dodecyl sulfate (SDS) were purchased from Sigma.

1.2. Instrument and Analysis

Proton and carbon-13 ($^1\text{H-NMR}$ and $^{13}\text{C-NMR}$) nuclear magnetic resonance spectroscopy (Bruker DPX-400/600) were used to determine the chemical structure of the synthesized polymers. Samples were dissolved at 5 mg/mL concentration in D_2O or $(\text{CD}_3)_2\text{SO}$ solvents depending on the solubility of the samples.

Size-exclusion chromatography (SEC) measurements were conducted on an Agilent 1260 infinity system operating in DMF with 5.0 mM NH_4BF_4 and equipped with refractive index detector (RID) and variable wavelength detector (VWD), 2 PLgel 5 μm mixed-C columns (300 \times 7.5mm), a PLgel 5 mm guard column (50 \times 7.5mm) and an autosampler. The instrument was calibrated with linear poly(methyl methacrylate) standards in range of 550 to 46890 $\text{g}\cdot\text{mol}^{-1}$. All samples were passed through 0.2 μm PTFE filter before analysis.

Thermal gravimetric analysis (TGA) was conducted with a TA Instruments TGA Q500 under nitrogen atmosphere using approximately 5.0 mg of the respective sample for the analysis. Method settings: heating from 100 to 900 $^\circ\text{C}$ with a heating rate of 10 $^\circ\text{C}/\text{min}$. UV measurements were performed on a PerkinElmer UV/Vis Spectrometer Lambda 35.

Transmission electron microscopy (TEM) analysis was carried out on a JEOL 1400 instrument operating at an acceleration voltage 100 kV. The TEM specimens were prepared by placing a drop of a nanoparticle water suspension on a carbon-coated copper grid.

UV-visible spectra were recorded on a PerkinElmer Lambda 25 UV/VIS spectrometer equipped with a (PTP-1) temperature control unit at a certain temperatures in the range of 200 nm and 600 nm using quartz microcuvettes.

Electrospray ionization-mass spectrometry (ESI-MS) spectra were recorded on a Thermo Finnigan LCQ Decaquadropole ion trap mass spectrometer (Thermo Finnigan, San

Jose, CA), equipped with an atmospheric pressure ionization source operating in the nebulizer assisted electrospray mode and was used in positive ion mode.

Spectrophotometric analysis was carried out to confirm the conjugation of DOX to particles, particle size, and surface charge. Size distribution and zeta potential of bioconjugates were measured by a dynamic light scattering (DLS) method with Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.) at a scattering angle of 90° using a wavelength of 633 nm and at 25 °C. Prior to measurements, the samples (50 µL) were diluted to 1.0 mL with PBS, and each sample was measured three times. Zeta potential of samples was calculated by the device according to Smoluchowski equation. The samples were kept in +4 °C when not in use. Spectrophotometric characterization of mannose tagged polymer conjugates were assessed *via* a UV-Vis Microplate Spectrophotometer (Epoch Microplate Spectrophotometer, Biotek).

1.3. Synthesis of D-mannose methacrylate glycomonomers

1-(2'-propargyl) D-mannose (2.61 g, 12.1 mmol) and 3-azidopropyl methacrylate (2.00 g, 11.0 mmol) were dissolved in MeOH/H₂O (3:1 vol/vol, 80 mL), aqueous solution of CuSO₄·5H₂O (240 mg, 0.97 mmol) and (+)-sodium L-ascorbate (250 mg, 1.22 mmol) were added into the reaction solution. The reaction mixture was stirred at ambient temperature for 24 h. Methanol was then removed under vacuum and the residue mixture was freeze dried to remove water. The obtained product was purified through silica gel column chromatography using dichloromethane-MeOH (8:1) as eluent. After removing of solvent, the product was obtained as white (1.94 g, yield: 43.9%).

¹H NMR (D₂O, 298 K, 400 MHz): δ =8.02 (s, 1 H, NCH=C), 5.96 (s, 1H, =CH₂), 5.58 (s, 1H, CH₂), 4.92-4.97 (m, 2H, CH₂-OH), 4.88-4.22 (m, CH-O-CH₂, H-1 of mannose , overlap

with H₂O), 4.54 (t, 2 H, J=6.6 Hz, CH₂-N), 4.21 (t, 2 H, J=6.8 Hz, C=O-O-CH₂), 3.51-3.92 (m, H residues of mannose), 2.26 (m, 2H, CH₂-CH₂-CH₂), 1.84 (s, 3H, CH₃) ppm.

¹³C NMR (D₂O, 298 K, 400 MHz): δ =158.4 (C=O), 134.6 (C-CH₂), 126.4 (N-CH=C), 123.7 (CH₂-C), 104.6 (β anomeric, C 1 of mannose), 103.2 (α anomeric, C 1 of mannose), 72.3 (N-CH=C), 71.6 (C=O-O-CH₂), 70.4 (C-CH₂-O), 66.8, 64.4, 63.5, 62.3 (carbons of anomeric mannose), 48.6 (CH₂-N), 29.7 (CH₂-CH₂-N), 19.4 (CH₂-OH), 18.6.5 (CH₃-C) ppm.

ESI-MS m/z: calculated for C₁₆H₂₅N₃O₈ (M+H⁺), 387.2; found, 387.4.

1.4. General procedure for RAFT polymerization

RAFT polymerization reactions were carried out in the presence of CPDBT as a RAFT agent, AIBN as an initiator in the methanol and water mixture (2:1) at 70 °C for 12 h. A Schlenk tube was charged with targeted monomer or monomers (methacrylic acid (MAA), poly(ethylene glycol) methyl ether methacrylate (OEGMA) and D-mannose methacrylate (ManMac)) (in total 100 eq), CPDBT (1 eq), AIBN (0.1 eq) and the solvent (3.0 mL) was degassed by gentle bubbling of argon gas for 30 min. The Schlenk tube was sealed properly and the mixed solution was allowed to polymerize. After the confirmation of nearly full conversion of monomers according to ¹H NMR, the polymerization reaction was stopped by cooling down and exposure to the air. Subsequently, the reaction solution was diluted with 3.0 mL of MeOH or H₂O and then purified by precipitation in diethyl ether or dialysis against distilled water. After the purification, the obtained polymer was dried under vacuum or by freeze-dryer and characterized *via* ¹H-NMR and DMF SEC analysis.

1.5. Reduction of the RAFT End Group of the obtained polymers

The RAFT end group of the obtained polymers was reduced by the addition of NaBH₄ as the reducing agent in distilled water. The homo/co-polymers were added into a 50-mL round-

bottom flask with 10 mL water solution of 1.0 M NaBH₄ and the solution was bubbled for 15 min with argon (molar ratio of NaBH₄:dithioester end groups was 25:1). After that, the mixture was allowed to react for 2 h. Following reduction, the reaction solution was dialyzed against distilled water for 3 days, while changing the water at least three times. Finally, it was freeze dried to get the polymer with thiol end group. The product was characterized by ¹H-NMR and DMF SEC analysis.

1.6. Preparation of Polymer-Substituted AuNPs

The AuNPs solution was centrifuged in order to remove the supernatant and then replaced by the same volume of water prior to the polymer functionalization. The terminally thiolated polymers (10 mg) were dissolved in 1.0 mL AuNPs solution and then agitated in the dark for overnight. To remove excess polymer, the solution of the AuNPs-stabilized polymers were centrifuged (5470 rpm, 30 min). Following careful decantation of the supernatant, the nanoparticles were then redispersed in 1.0 mL of deionized water and then the centrifugation and resuspension process was repeated one more cycle. These synthesized polymers-substituted GNPs were characterized by DLS, UV/Vis Spectroscopy, TGA and TEM. The polymer-coated AuNPs were stored at 4 °C for the further study.

1.7. Preparation of pH-Sensitive Bioconjugates

Four different bioconjugates within DOX were synthesized and coded as P1D (AuNP-P(MAA)₉₆-DOX), P2D (AuNP-P((MAA)₄₄-r-(ManMac)₄₆)-DOX), P3D (AuNP-P((OEGMA)₄₃-r-(ManMac)₄₅)-DOX) and P4D (AuNP-P((ManMac)₉₄)-DOX).

Preparation of P1D. pH-sensitive hydrazone bond between DOX molecule and PMAA coated AuNPs (P1D) were performed according to our previous work.¹ In brief, AuNPs that have pendant carboxylic acid (–COOH) groups *via* PMAA structure were activated with

EDC/NHS chemistry, and covalent amide bonds were generated by adding Cys. For this purpose, the mixture of 25 μL of PMAA-AuNP (18 mg/mL dissolved in 10 mM phosphate buffer, pH 7.4), 48.9 mg EDC, 7.2 mg NHS, and 250 μL of Cys (from 1.0 mg/mL stock solution dissolved in pH 5.0 MES buffer) was prepared ($V_{\text{total}} = 1500 \mu\text{L}$ with the addition of MES buffer) and incubated for 3 h with 1000 rpm shaking under ambient conditions. After the incubation step, a final mixture was dialyzed against pH 7.4 PBS for 2 h. The dialyzed bioconjugate solutions were treated with DTT (1:0.9 molar ratio of Cys/DTT). The mixtures were incubated for 4 h under 1000 rpm shaking and ambient conditions. After the reduction of S–S linkages between Cys residues, EMCH cross-linker was added as 3.2 mg and reacted for 2 h at 1000 rpm shaking at room temperature. Following the incubation, the obtained solutions were again dialyzed overnight against PBS pH 7.4. In the final step, DOX (50 μM as the final concentration) was added to the Cys modified pre-conjugate (EMCH) solutions and incubated for 2 h at 1000 rpm shaking and room temperature and final solution was increased to 2.0 mL. The final conjugates were dialyzed overnight against PBS, pH 7.4.

Preparation of P2D, P3D and P4D. On the other hand, the bioconjugation of mannose tagged and polymer covered AuNPs (P2D, P3D and P4D) is carried out as three main steps. Initially, AuNPs that have pendant hydroxyl (-OH) *via* Man terminal molecule were activated with CDI coupling, and covalent amide bonds were generated by adding Cys. This step is the main difference from P1D synthesis which includes EDC/ NHS coupling between carboxyl groups of PMAA and amino groups of Cys. For the purpose of CDI coupling, the mixture of 500 μL of Man tagged AuNP (0.4 mg/ mL (P2 and P3), 10 mg/mL (P4) dissolved in 10 mM phosphate buffer, pH 7.4) and 250 μL of CDI (from 12 mg/mL stock solution dissolved in DMSO) was prepared and incubated for 2 h with 1000 rpm shaking at 37°C to activate –OH groups. After the activation step, 250 μL of Cys (from 1.0 mg/mL stock solution dissolved in pH 8.5 Borate buffer) was added to reaction mixture and incubated overnight under 1000 rpm

shaking under ambient conditions ($V_{\text{total}}=1500 \mu\text{L}$ with the addition of borate buffer). After the incubation time, a final mixture was dialyzed against pH 7.4 PBS for 2 h. Following steps are carried out in the same way as P1D preparation after first dialysis.

1.8. *In Vitro* Drug Release

The drug release behaviours of AuNP core-shell polymer conjugates was monitored by creating artificial media. pH values of 7.4 and 5.3 (PBS) were used to simulate the healthy and cancerous cellular environments, respectively. Dialysis bags containing 0.5 mL of sample were immersed in 5.0 mL of buffer medium at 37 °C at 100 rpm. To investigate the *in vitro* release profiles of the samples, 0.5 mL of each of the samples was collected at several time intervals (0 and 30 min and 1, 2, 4, 6, 8, 12, 24, 48, and 72 h) and replaced with an equal volume of fresh medium. The concentration of DOX in the collected samples was determined spectrophotometrically by using a standard curve that was generated by the absorbance of DOX. Probing the released DOX, the cumulative drug release percentage (E_r) was calculated according to the following equation:

$$E_r = \frac{V_e \sum_{i=1}^{n-1} C_i + V_o C_n}{M(\text{Dox})}$$

where $M(\text{Dox})$ represents the amount of DOX in the particles, V_o is the whole volume of the release media ($V_o = 5.0 \text{ mL}$), V_e is the volume of the replace media ($V_e = 0.5 \text{ mL}$), and C_n represents the concentration of DOX in the n th sample.²

1.9. Cell Culture

Human cervix adenocarcinoma (HeLa), human lung carcinoma (A549), human neuroblastoma cells (SH-SY5Y), and monkey kidney epithelial cell line (Vero) were

maintained in DMEM supplemented with 10% FBS, 100 UI/mL penicillin/streptomycin, and 2.0 mM L-Glutamine at 37 °C in a humidified incubator with 5.0% CO₂. Cells were subcultured at 80% confluency by trypsinization. Polymers and bioconjugates were dissolved in DMEM in all experiments.

1.10. Cell Viability

Colorimetric MTT assay was used to assess relative cell viability. Briefly, 10,000 cells/well were incubated in 96-well cell culture plate for 24 h at standard culture conditions. AuNPs (P1-P4) were instructed to cells at concentrations between 5-250 µg/mL and incubated for 24 h. DOX and DOX conjugated AuNPs (P1D, P2D, P3D, P4D) were applied at doses between 0.01 - 1.00 µM (equivalent DOX) and incubated for 24 h. After desired incubation time with polymers and bioconjugates, MTT solution (0.5 mg/mL in DMEM) was added to each well and incubated for 4 h. Intracellular formazan crystals produced by the enzymatic activity of living cells were dissolved in 10% SDS (in 0.01 M HCl) and quantified by reading the absorbance at 570 nm. Absorbance at 620 nm was used as reference wavelength.

DMEM without any sample was used as control and considered as 100% viable. Relative cell viability was plotted as the percent absorbance of sample treated cells.

1.11. Confocal Microscopy Imaging

SH-SY5Y cells were plated on 6-well plate (100,000 cells/well) and incubated for 24 h before sample application. Then, medium was aspirated and cells were incubated with DOX conjugated AuNPs (P1D, P2D, P3D and P4D) and free DOX (equivalent DOX concentration 0.5 µM) for 3h. Images were obtained with an Andor Revolution Confocal Laser Microscope (Olympus IX-71 fluorescence microscopy) with the exposure of 488 nm laser.

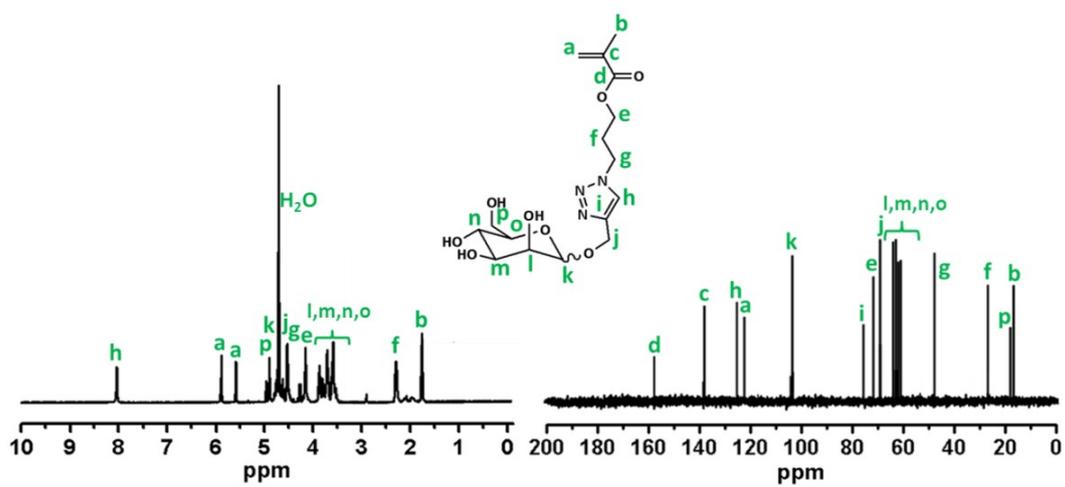


Figure S1. Details of ^1H -NMR and ^{13}C -NMR spectrum of D-mannose methacrylate glycomonomer.

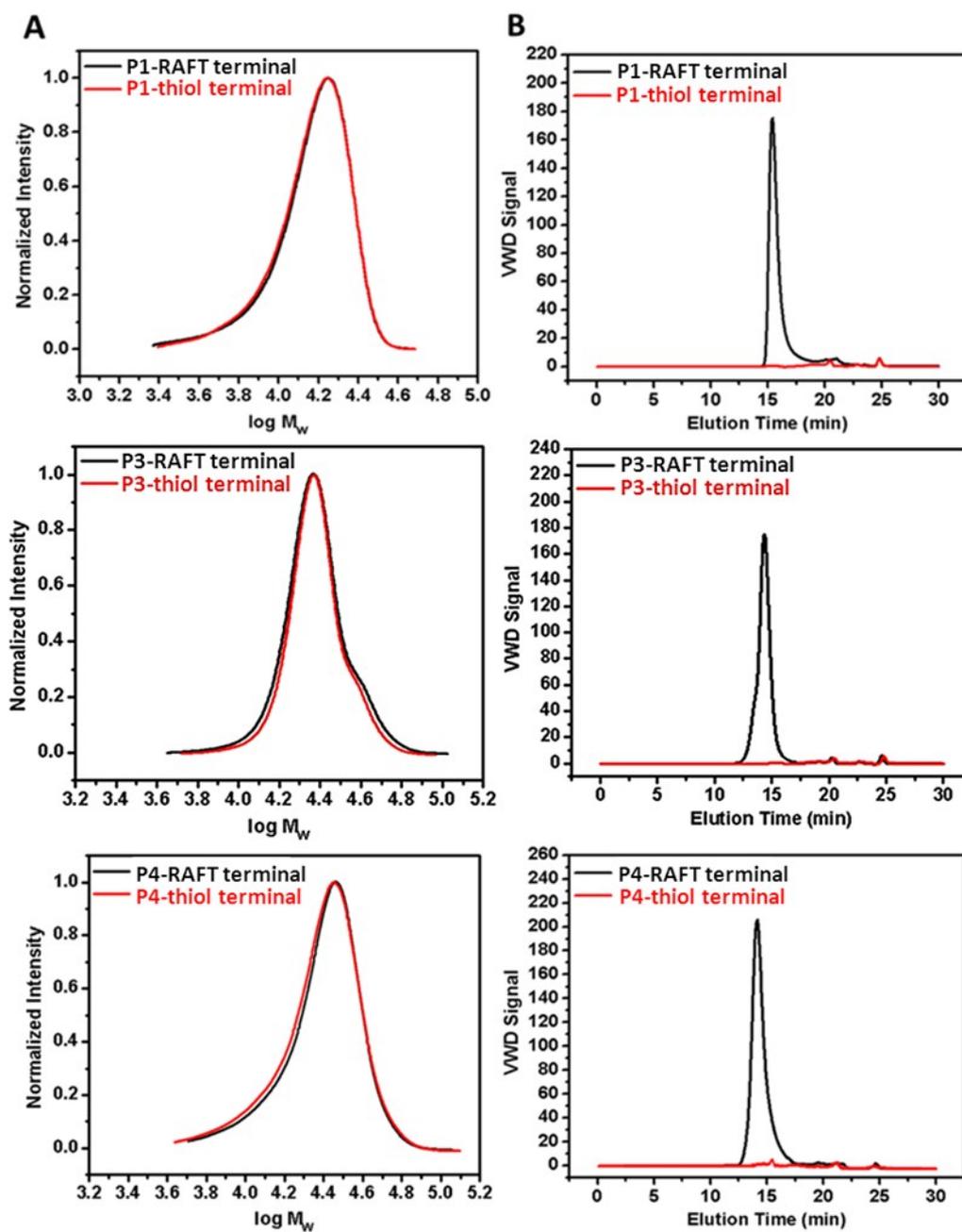


Figure S2. SEC analysis *via* RI (A) and VWD (B) detector before and after the reduction of the RAFT terminal group.

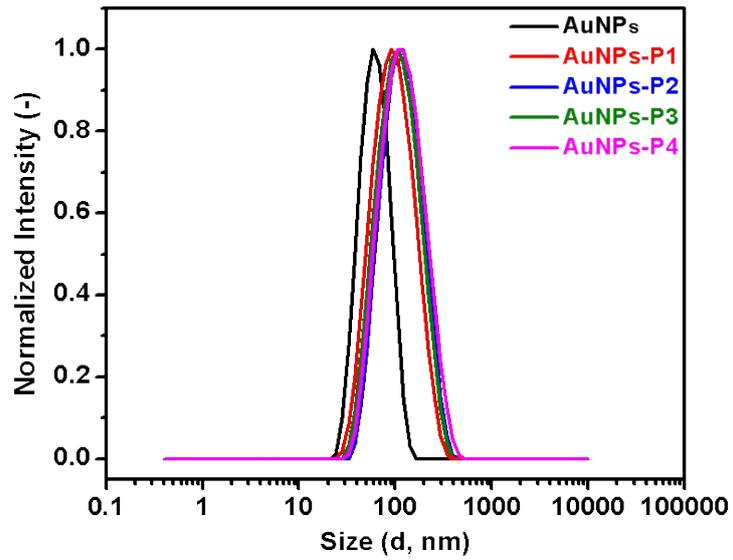


Figure S3. DLS measurements of the synthesized polymer-coated AuNPs particles.

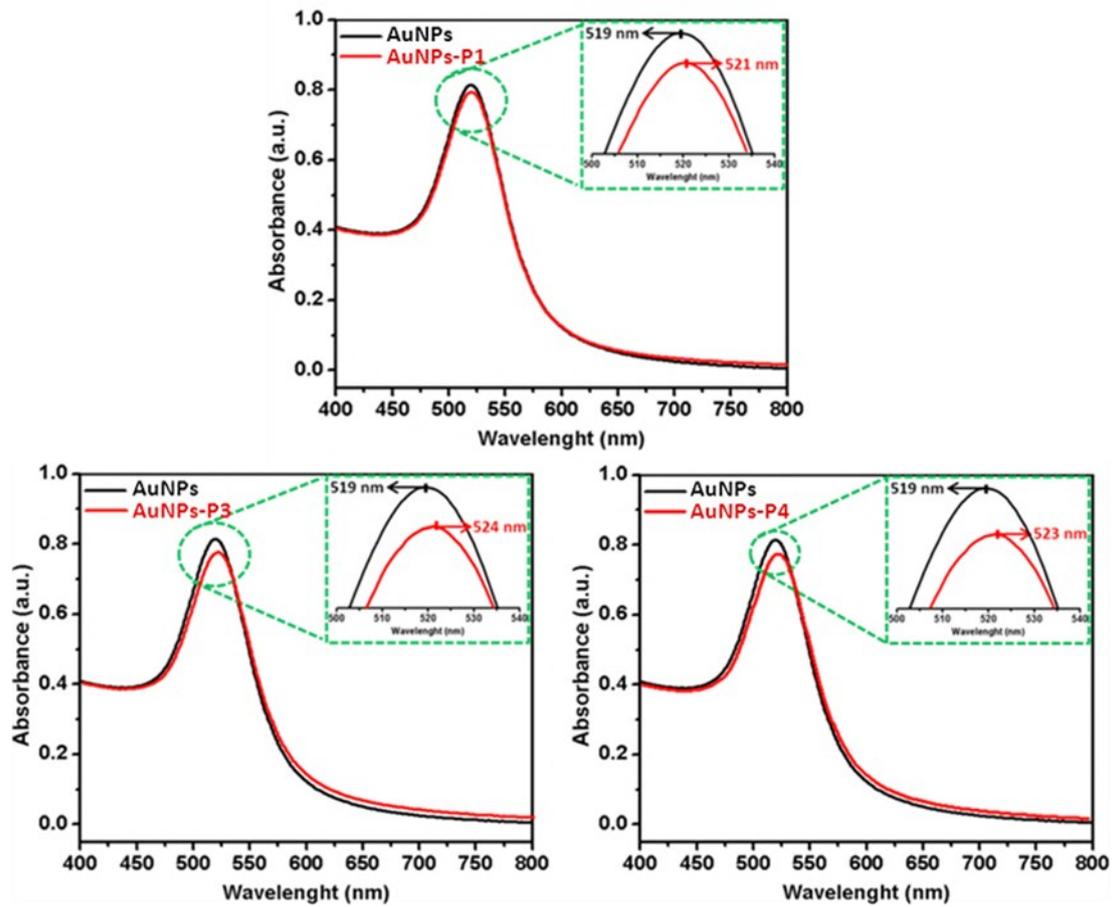


Figure S4. UV/Vis spectroscopy characterization of AuNPs and the obtained glycopolymer-coated AuNPs particles.

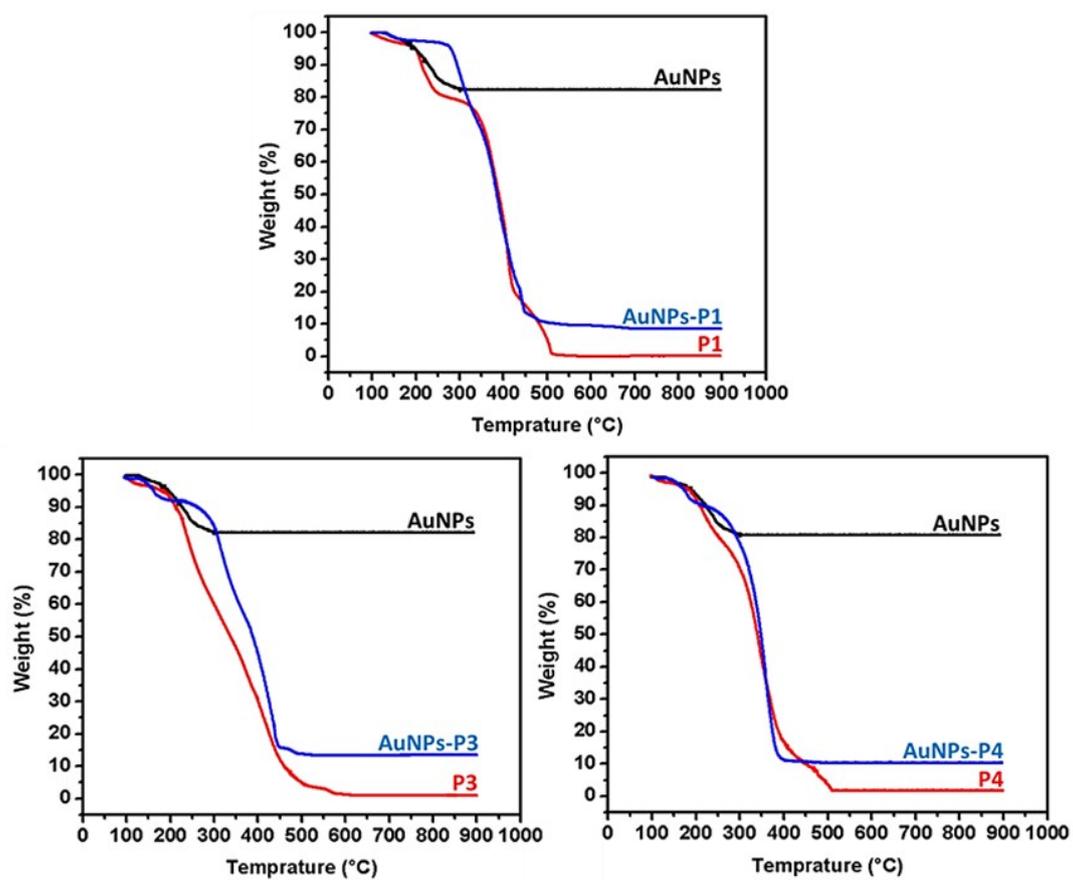


Figure S5. TGA measurements of AuNPs, all synthesized glycopolymers and their substituted AuNPs.

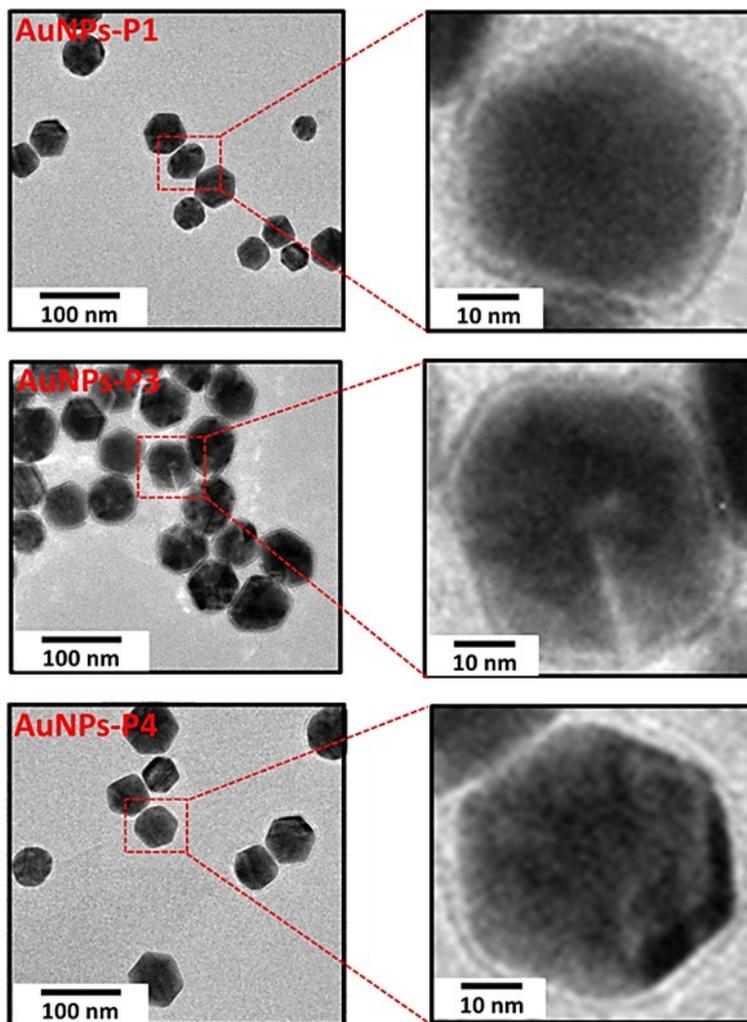


Figure S6. TEM images of the obtained glycopolymer-coated AuNPs particles.

References

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- (2) Chang, L.; Deng, L.; Wang, W.; Lv, Z.; Hu, F.; Dong, A.; Zhang, J. *Biomacromolecules* **2012**, *13* (10), 3301–3310.

