Facile Preparation of Glyconanoparticles and Their Bioconjugation to Streptavidin

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Well-defined glycopolymers containing linear and cyclic carbohydrate moieties as pendent groups were prepared by reversible addition fragmentation chain transfer polymerization (RAFT). The RAFT synthesized glycopolymers were used for the aqueous synthesis of stabilized glyconanoparticles. The in situ reduction of the glycopolymers and $HAuCl_4$ resulted in the formation of highly stable modified gold nanoparticles with diameters ranging from 40 to 80 nm in aqueous media. Multifunctional glyconanoparticles were also generated in the presence of varying amounts of biotinylated-polyethyleneglycol (*bio*-PEG-SH) having terminal thiol groups. The gold nanoparticles underwent aggregation in the presence of streptavidin as revealed by UV−vis spectroscopy. The availability of the biotin for conjugation to streptavidin was also confirmed using surface plasmon resonance (SPR).

Introduction

Recent advances in nanotechnology have opened up a wide range of highly sophisticated and promising biorelated applications for smaller and smarter functionalized nanoparticles.[1] Nanoparticles with tailored properties and flexible surface functionalization can be used to generate a multifunctional platform for various biomedical applications.[2] Surface properties and compositions of the nanoparticles are key issues to address for application in complex biosystems. Ideally, surface modification of nanoparticles should provide good colloidal stability, biocompatibility, and specific biorecognition of the particle surface.[3] Gold-based nanoparticles have been widely used in drug delivery, biodetection, and medical imaging due to their optical properties, their electrochemical properties, and their ease of functionalization.[4] Thiol chemistry has been widely applied to the surface modification of gold nanoparticles ranging from small molecules, to synthetic polymers, to biomacromolecules species.[5−17] To date, most biomedical applications rely on the use of poly(ethylene glycol)-modified (PEGylated) gold nanoparticles. The hydrophilic PEG serves as a protective shield to prevent nonspecific interactions. These functionalized nanoparticles have proved to be very stable in biosystems and do not have any deleterious effect. Recently, gold nanoparticles stabilized with carbohydrate moieties have been synthesized and applied as model systems in glycobiology and also as antiadhesion agents in potential tumor treatment.[18−20] The presence of the carbohydrate units has a dramatic effect on the physical, chemical, and biological properties of the glycoproteins and the latter has broad functions as markers in cell−cell communication events that determine microbial virulence,[21] inflammation,[22,23] and host immune response.[24−27] Thus, the design of synthetic glyconanoparticles having cyclic carbohydrate units that resemble the

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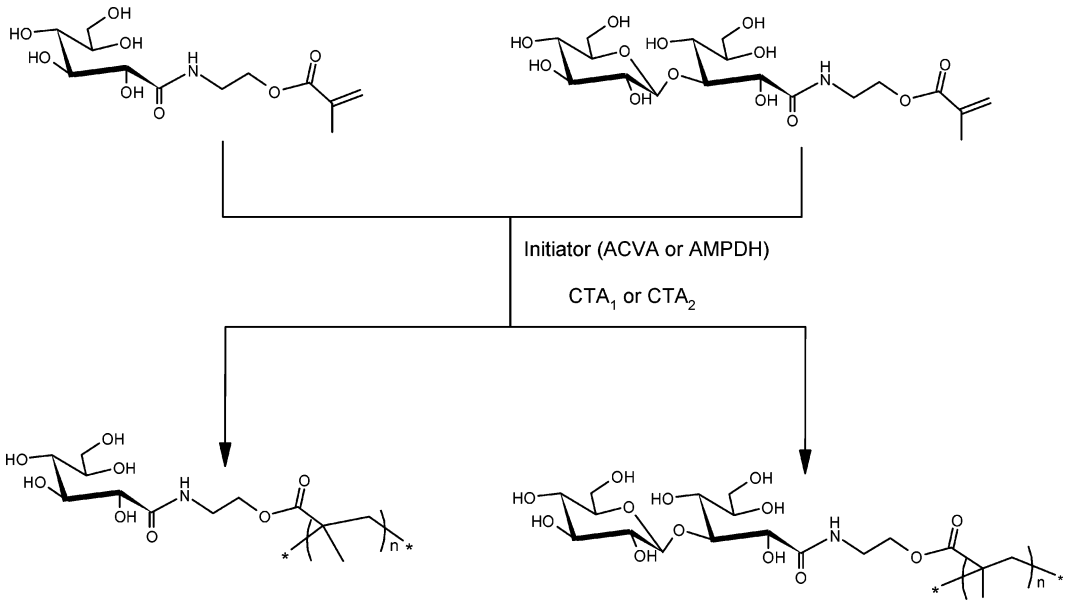
Scheme 1. RAFT Polymerization of the Glycomonomers GAMA and LAMA[a]



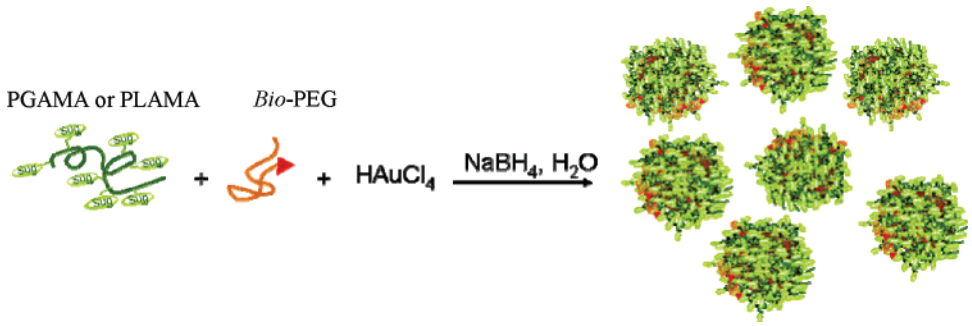
[a] CTA_1: *S*,*S*′-bis (α,α′-dimethyl- α″-acetic acid)-trithiocarbonate CTA_2: 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid ACVA: 4,4′-azobis(4-cyanovaleric acid) AMPDH: 2,2′-Azobis(2-methylpropionamidine) dihydrochloride.

Table 1. Synthetic Parameters for the Homopolymerization of the Glycomonomers GAMA and LAMA

sample	solvent composition	initiator	CTA[a]	temp. (°C)	CTA:initiator	conversion[c] (%)	M_n[b]	M_w/M_n
$PGAMA_1$	H_2O/DMF (3:2)	ACVA	CTA_1	60	5	>95	14,070	1.19
$PGAMA_2$	H_2O/DMF (3:2)	ACVA	CTA_2	65	15	>95	21,200	1.25
$PGAMA_3$	H_2O/MeOH (4:1)	ACVA	CTA_2	60	5	>95	51,550	1.48
$PLAMA_4$	H_2O/DMF (5:1)	ACVA	CTA_1	60	5	>95	24,730	1.22
$PLAMA_5$	H_2O/DMF (4:1)	AMPDH	CTA_2	65	5	>95	45,976	1.25

[a] Chain transfer agent. [b] Determined by gel permeation chromatography using water containing 0.1 M sodium nitrate as eluent and polyethylene oxide as calibration standards. [c] The conversions were determined by ^1H NMR and were found to be higher than 95%. However, after purification, the glycopolymers were obtained in 75−80%.

Scheme 2. Formation of Stable Biotinylated Glyconanoparticles



naturally occurring glycoproteins can potentially be very useful in human therapeutics and drug discovery applications and can exhibit increased specificity in carbohydrate−protein recognition events in biological systems. Recently, there has been increasing attention paid to synthetic polymers with pendent saccharide moieties, so-called glycopolymers, as biological recognition agents. With the advent of living radical polymerization techniques (LRP),[28−29] glycopolymers of controlled dimensions (narrow polydispersity and predetermined molecular weights) have been prepared without using any protecting group chemistries. LRP techniques such as reversible addition fragmentation chain transfer (RAFT) and atom transfer radical polymerizations (ATRP) are tolerant to a wide range of functional groups which has allowed the facile preparation of tailor-made glycopolymers.[30−38] Since our main interest is to design functionalized gold nanoparticles, the RAFT technique was selected to synthesize the glycopolymers.

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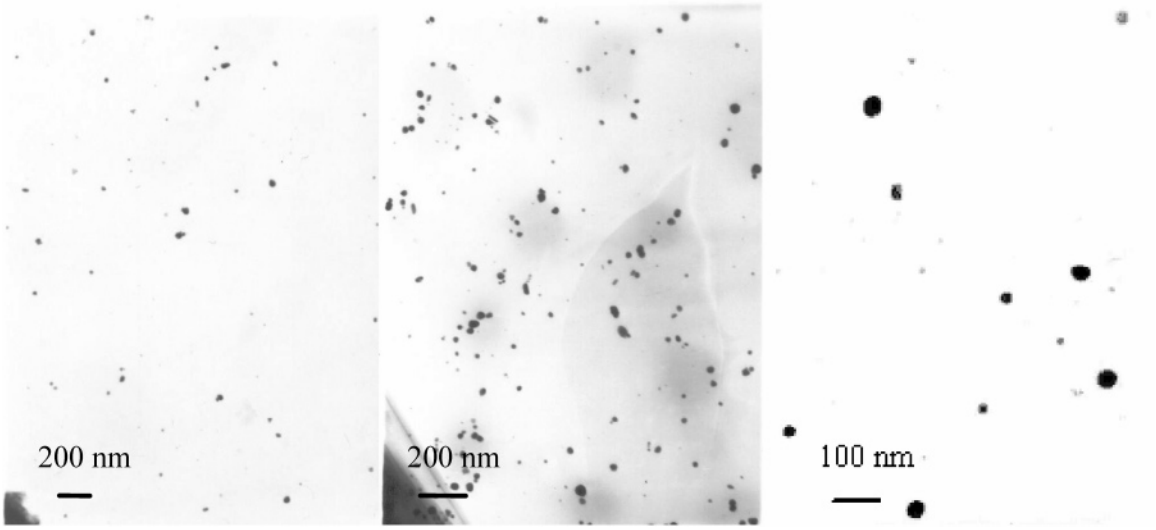

Figure 1. Transmission electron micrographs for the formation of glyconanoparticles (entry A-4).

Scheme 3. Mechanism for the Bioconjugation of Biotinylated Gold Glyconanoparticles with Streptavidin

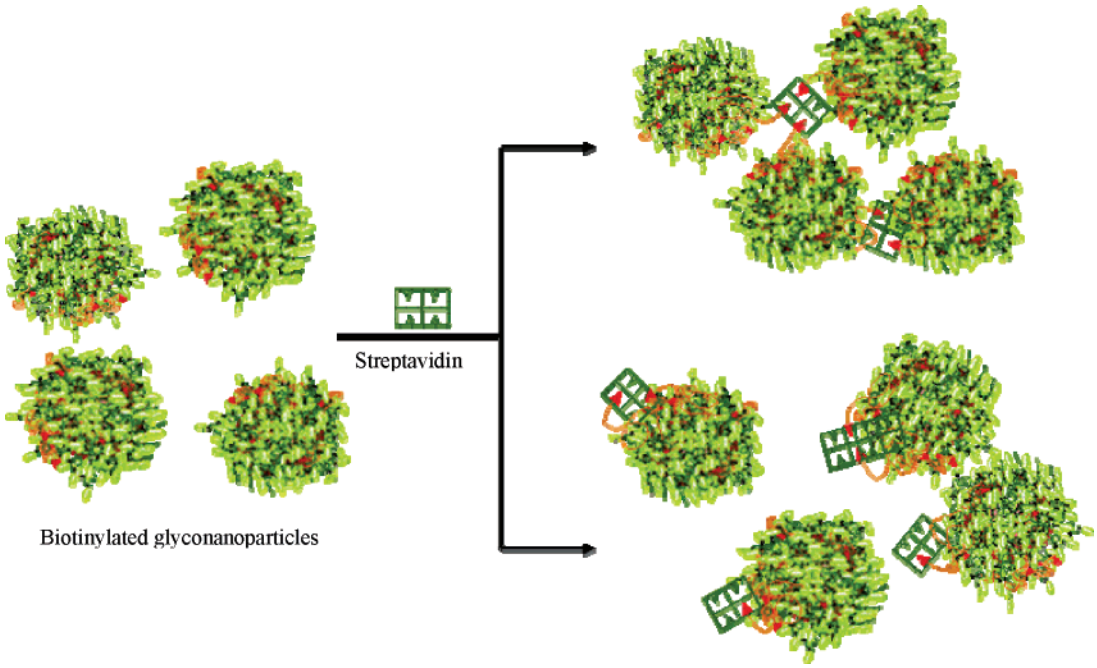

Table 2. Composition and Sizing Data for the Glyconanoparticles

gold nanoparticles samples	composition	molar ratio	average diameter (nm, DLS)	polydispersity index (PDI)
A-1	P(LAMA$_1$)		78.6	0.26
A-2	P(LAMA$_1$)/*bio*-PEG	80/20	68.3	0.21
A-3	P(LAMA$_1$)/*bio*-PEG	90/10	71.5	0.13
A-4	P(GAMA$_2$)		60.8	0.25
A-5	P(GAMA$_3$)/*bio*-PEG	80/20	43.9	0.18
A-6	P(GAMA$_3$)/*bio*-PEG	90/10	53.3	0.16

Here, we describe the synthesis of novel, well-defined glycopolymers containing linear and cyclic carbohydrate moieties prepared by RAFT polymerization (RAFT). The glycopolymers were used in the synthesis of stabilized glyconanoparticles in aqueous solution. The facile in situ reduction of the RAFT prepared glycopolymers and $HAuCl_4$ resulted in the formation of highly stable gold sol in aqueous solution. Multifunctional glyconanoparticles were also generated in the presence of a varying amount of biotinylated-polyethyleneglycol having terminal thiol groups as a model bioactive domain. Addition of streptavidin to the biotinylated glyconanoparticles resulted in particle aggregation, thus confirming the accessibility and recognition of the biotin moieties.

Experimental Section

Materials. All chemicals were purchased from Sigma-Aldrich, Anachemia, or Acros Chemicals and were used without further purification. 2-Aminoethyl methacrylate (AMA), D-gluconamidoethyl methacrylate (GAMA), and 2-lactobionamidoethyl methacrylate (LAMA) were synthesized according to the method reported previously[30−32] and highly efficient chain transfer agent *S*,*S*′-bis-(α, α′-dimethyl-α″-acetic acid)-trithiocarbonate (CTA$_1$) was prepared according to the method described by Lai et al.[39]

Polymer Characterization. Molecular weight and molecular weights distribution of the polymer was determined by using gel permeation chromatography (GPC) using water as eluent. The following protocols were used in this work. The polymers were analyzed using a Viscotek GPC instrument. The system comprises

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of a triple detector (refractive index detector, light scattering, and viscometer), mixed Viscogel columns, and water containing 0.1 M KNO_3 was used as eluent at a flow rate of 1.0 mL min^{-1}. The GPC calibration was achieved using a series of monodisperse polyethylene glycol (PEG) and polyethylene oxide (PEO) standards. The molecular weights and polydispersity data were calculated using the OmniSEC 4.1 software package. The ^1H NMR spectra were recorded in D_2O using a 200 MHz Varian spectrometer.

Typical RAFT Polymerization of D-Gluconamidoethyl Methacrylate (GAMA). d-Gluconamidoethyl methacrylate (250 mg, 0.81 mmol) was dissolved in a degassed mixture of water and *N*,*N*′-dimethylformamide (DMF) (3:2 v/v). A mixture of *S*,*S*′-bis-(α,α′-dimethyl-α″-acetic acid)-trithiocarbonate (CTA1) (2.8 mg, 6 μmol)/4,4′-azobis(4-cyanovaleric acid) (ACVA) (0.2 mg, 0.6 μmol) was added, and the solution was purged under nitrogen. The tube was then sealed under inert atmosphere, and the polymerization reaction was carried out at 60−65 °C for 18 h. After that time, the polymer solution was cooled to room temperature and then precipitated with methanol. The white precipitate formed was filtered and dried under vacuo. The polymer was further dissolved in 5 mL of water, and the solution was freeze-dried overnight. A creamy white flaky solid was obtained.

A similar experimental protocol was followed for the homopolymerization of 2-lactobionamidoethyl methacrylate (LAMA).

Typical Synthesis of PGAMA-Coated Au Nanoparticles. To a test tube was added PGAMA (198.3 mg, $M_n = 21\,200$) to a solution of $HAuCl_4$ (10 mg/mL). The mixture was magnetically stirred to ensure homogeneity at room temperature. Then aqueous sodium borohydride solution (1 M, 2.0 mL) was added dropwise, and the solution change rapidly from pale yellow to dark red indicating the formation of the gold colloids. The color change is due to size dependence surface plasmon resonance properties of gold metal. The mixture was allowed to continue stirring at room temperature for 20 h. The solution was then filtered with a 0.45 μm filter, and the mixture was purified by dialysis using a dialysis membrane (molecular weight cutoff, MWCO = 3500). The PGAMA gold colloids were monitored for several weeks and were found to be very stable similar to synthesized pegylated gold colloids.[4a]

A similar procedure was followed for the synthesis of the PLAMA coated gold nanoparticles.

Dynamic Light Scattering Measurements. Dynamic light scattering experiments were performed on a Viscotek DLS instrument containing a He−Ne laser at a wavelength of 632 nm.

Transmission Electron Microscopy (TEM). TEM analyses were conducted on a Hitachi 800 transmission electron microscope at an acceleration of voltage of 200 kV. The samples for TEM were prepared by placing a drop of the nanoparticle solutions on carbon coated copper grids.

Surface Plasmon Resonance Measurements. A Biacore X SPR was used to study the binding events between the biotinylated magnetic nanoparticles and the surface immobilized streptavidin sensor chip. The buffer (HEPES − 0.01 M, 0.15 M NaCl, 0.005% surfactant P20) and SA sensor chip was purchased from Biacore Inc. The flow rate was set to 10 or 20 μL/min, and between 25 and 50 μL of sample was injected at a time.

Results and Discussion

We describe here reversible addition fragmentation chain transfer (RAFT) polymerization of the glycomonomers, 2-gluconamidoethyl methacrylate (GAMA), and 2-lactobionamidoethyl methacrylate (LAMA), using two different chain transfer agents and two different initiators as shown in Scheme 1. The RAFT polymerization technique was selected first because it is a robust technique for the polymerization of glycomonomers in aqueous media. Second, telechelic glycopolymers can be generated where the RAFT agent can be easily cleaved for the stabilization of gold nanoparticles. The syntheses of the glycomonomers, GAMA, and LAMA, were previously reported.[30−32] These monomers were prepared by a very facile synthetic approach without using any protecting group chemistry. Due to the presence of the unprotected hydroxyl groups of the carbohydrate moieties, it would be more interesting to investigate the RAFT polymerization in water. However, due to the low solubility of the chain transfer agents in pure water, the polymerization was conducted in mixtures of water and *N*,*N*′-dimethylformamide (DMF) or methanol. The target degree of polymerization was set to 50 or 100, and the ratio of the chain

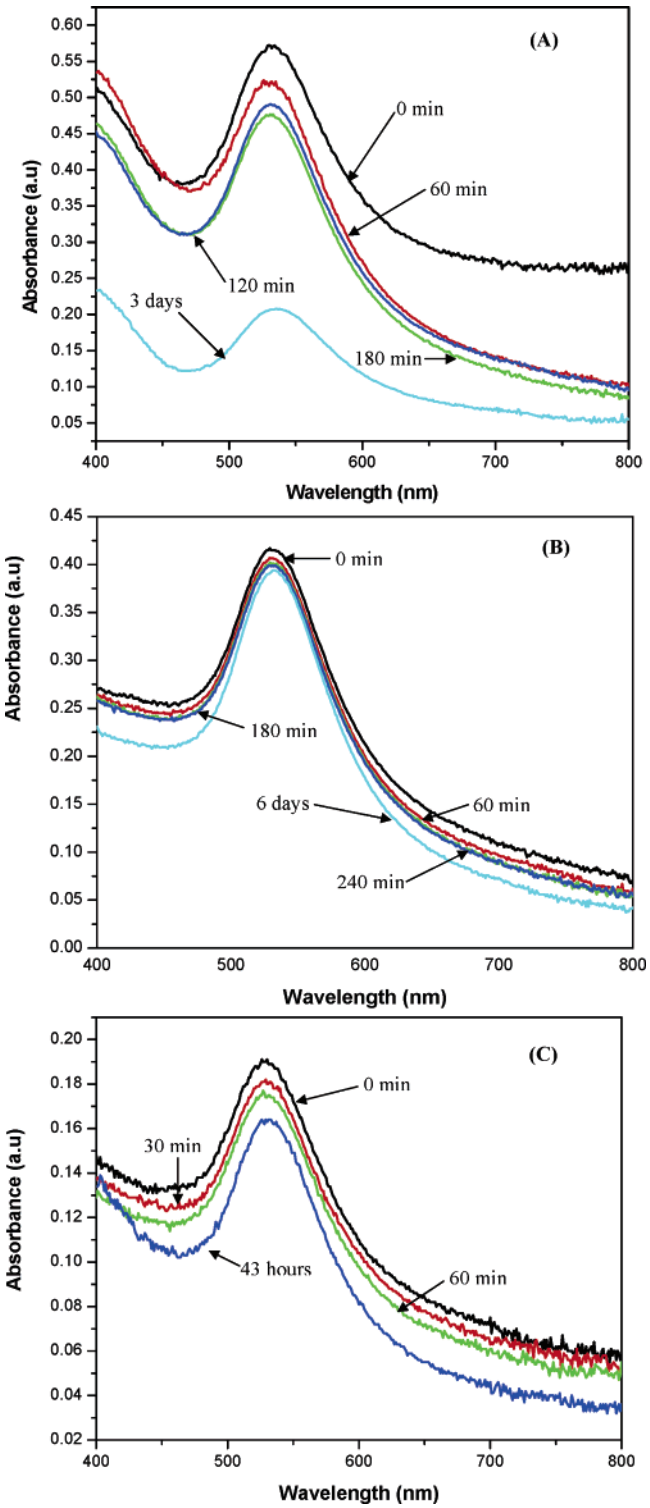

Figure 2. Change of absorption with time after addition of 100 μg/mL of streptavidin to (A) p($GAMA_3$)/biotin-PEG (8:2) (Table 2, sample A-5); (B) p($GAMA_3$)/biotin-PEG (9:1) (Table 2, sample A-6); (C) p($LAMA_1$)/biotin-PEG (8:2) gold nanoparticles (Table 2, sample A-2).

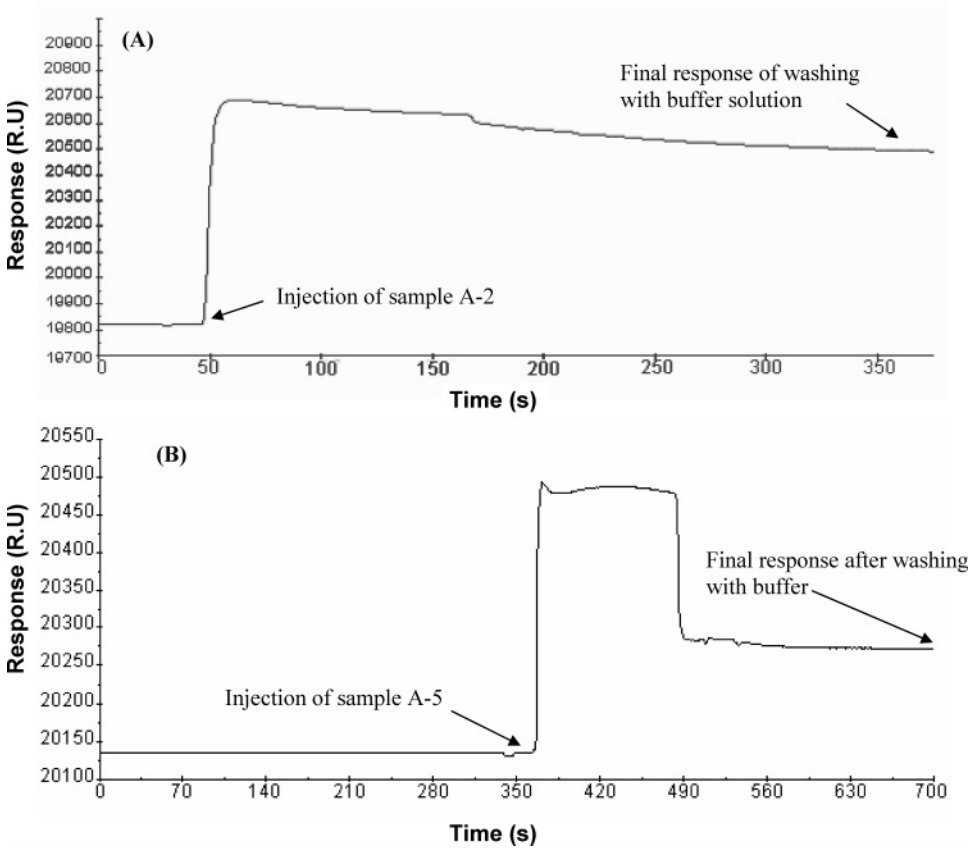

Figure 3. SPR sensorgram for the bioconjugation of the biotinylated glyconanoparticles to streptavidin coated sensor chip: response after sample A-2 injection **(**A**)** and response after A-5 injection. **(**B**)** Data obtained from a Biacore X instrument using HBS-EP buffer at a flow rate of 20 μL/min and temperature set at 25 °C.

transfer agent to initiator used was 5 or 15. After polymerization, a clear yellow viscous solution was obtained indicating the absence of any side reactions or gel formation. The synthetic parameters for the polymerization are indicated in Table 1. The polymer conversions were determined by ^{1}H NMR and were found to be higher than 95%. After purification and drying, the final yields of the glycopolymers were 75−80%. It should be noted that, when pure deionized distilled water was used as solvent, the polymerization of the glycomonomers is uncontrolled, most probably due to the very low solubility of the chain transfer agents. In all cases, the chain transfer agent was dissolved in DMF or methanol before addition to the polymerization mixture.

The glycopolymers were then evaluated for the synthesis of stabilized glyconanoparticles in aqueous solution. The well-established thiol-chemical method for gold stabilization is applied here for the formation of glycopolymer-stabilized gold nanoparticles. The in situ reduction of the RAFT-prepared glycopolymers and $HAuCl_4$ resulted in the formation of highly stable gold sol in aqueous solution. The glyconanoparticles were also generated in the presence of varying amounts of biotinylated-polyethyleneglycol having terminal thiol groups (*bio*-PEG-SH) as shown in Scheme 2. The motivation for biotinylation of the glyconanoparticles is to demonstrate the feasibility of the bioactive groups on the nanoparticles toward bioconjugation to a high affinity ligand such as streptavidin in solution as well when immobilized on a surface. Upon addition of sodium borohydride, the solution changed from pale yellow to reddish brown indicative of the formation of gold colloids. The red color of the solution is due to the size dependence of surface plasmon resonance of the gold colloids. The addition of the reducing agent causes the reduction of Au^{3+} to Au metal as well as generates the thiol in situ via the cleavage of the trithiocarbonates at the polymer chain ends. Scheme 2 shows the basic mechanism for the formation of thiol stabilized gold colloids. It should be noted that the in situ reduction minimizes the formation of disulfide linkages between the thiol terminated polymer chains. Each reaction solution was allowed to stir at room temperature for more than 24 h, prior to purification via dialysis. Particle size measurements of the gold sol were obtained by dynamic light scattering (DLS), and the data were collected before and after purification. The results are tabulated in Table 2. The sizes of the glyconanoparticles do not change over weeks indicative of the very high stability similar to PEGylated gold colloids.[4] The gold colloids were found to be very stable even in the presence of high salt concentration (2 M KCl). Thus, the glycopolymers can serve as a natural material-based alternative to PEGylation. The size and shape of the functionalized nanoparticles were examined by transmission electron microscopy (TEM). Figure 1 shows the spherical morphology of the nanoparticles for the p(GAMA)-Au sample. It should be noted the sizes of the glyconanoparticles ($PGAMA_2$-Au) are smaller (∼45 nm) which is indicative that only the gold core is visible by the TEM image. Dynamic light scattering indicates a larger size corresponding to the polymer chains on the gold nanoparticles.

Furthermore, we investigated the availability of the biotins incorporated on the surface of the nanoparticles for interaction with streptavidin. Streptavidin is one of the most widely used proteins in a number of applications such as in affinity separations, bioassays and clinical diagnostics because of its high affinity to biotin (10^{13-15} M^{-1}). It should be noted that biotin is connected to a very flexible PEG chain of molar mass 5 kDa and the molar masses of PGAMA and PLAMA are 48 and 49 kDa respectively. The molar ratio of the glycopolymers to PEG is varied as indicated in Table 2. The addition of streptavidin to the gold dispersions

was monitored with time spectrophotometrically to record the change of the plasmon absorption (see Figure 2). A shift of the maximum absorbance to lower values was noted with time indicating an aggregation of the gold nanoparticles. A bathochromic shift of the maximum wavelength was also noted and which is quite significant with the surface composition of the nanoparticles with P(LAMA-1)/*bio*-PEG (Table 2, sample A-2) or P($GAMA_3$)/*bio*-PEG (Table 2, sample A-5) of ratio 80/20. These results are consistent with the work of Ishii and co-workers[40] regarding the aggregation of bio-PEGylated gold nanoparticles in the presence of streptavidin. As a blank, nonbiotinylated glyconanoparticles were also synthesized and studied with streptavidin. No time dependent aggregation of the nanoparticles was observed spectrophometrically. This shows that the aggregation occurs only because of the biotin conjugation to streptavidin. Since the size of the gold nanoparticles are in the range of ∼50−80 nm as compared to the size of streptavidin which is ∼3−5 nm, the aggregation does not cause a dramatic bathochromic shift of the maximum wavelength. A very small bathochromic shift of the maximum wavelength is observed for a surface composition of 90/10: (P($GAMA_3$)/*bio*-PEG (Table 2, entry 6) or P($LAMA_1$)/*bio*-PEG) (Table 2, entry 3) which is indicative that the lower biotin content does not cause a major aggregation of the gold nanoparticles to cause a bathochromic shift or a decrease of the maximum absorbance. These results are further supported by the surface plasmon resonance (SPR) studies conducted with the biotinylated gold glyconanoparticles (see Figure 3). SPR is a very sensitive technique to study biomolecular events in the nanomolar range. SPR has been extensively used to study the binding events between biotinylated molecules or macromolecules to streptavidin as the affinity of biotin to streptavidin is strong. We investigated the ability of the biotinylated glyconanoparticles to bind to a streptavidin coated sensor chip. The biotinylated glyconanoparticles: P($GAMA_3$)/*bio*-PEG or P($LAMA_1$)/*bio*-PEG of ratio 80/20 (Table 2, samples A-5 and A-2) were injected into the SPR instrument containing a preloaded streptavidin-coated sensor chip. After injection, an immediate change in response was observed, and after automatic wash (with buffer), the difference in response is indicative of the binding of the biotinylated gold glyconanoparticles to the streptavidin on the sensor chip. It should be noted that in the case of samples P($GAMA_3$)/*bio*-PEG or P($LAMA_1$)/*bio*-PEG of ratio 90/10 (Table 2, entries 3 and 6) no binding of the nanoparticles was noted which is consistent with the UV−vis absorption results.

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Conclusions

Well-defined glycopolymers containing both linear and cyclic carbohydrate moieties have been successfully synthesized by the reversible addition fragmentation chain transfer polymerization under aqueous conditions. The facile preparation of stable gold glyconanoparticles by the in situ reduction of $AuCl_4$ and the glycopolymers is indicative of the telechelic nature of the glycopolymers. The surface functionalization of the gold nanoparticles has also been studied with a varying amount of biotinylated polyethylene glycol, and we demonstrated the bioconjugation of the biotin ligand installed on the surface of the nanoparticles to streptavidin both in solution and when immobilized on a surface. We have further demonstrated that the biotin ligand can also be subjected to steric factors toward binding to streptavidin.

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Supporting Information Available: ^1H NMR spectra of the glycopolymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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