Chapter 13

Synthetic Approach to Glycopolymer Base Nanoparticle Gold(I) Conjugate: A New Generation of Therapeutic Agents

Christian K. Adokoh, James Darkwa, and Ravin Narain

Abstract

Advances in nanotechnology have led to the fabrication of nano-constructs of organic or inorganic origins with well-defined structures, surface properties, and can be made to respond to physical or chemical stimuli. These nano-constructs can provide a shift in the way diagnostic and therapeutic drugs are delivered to achieve target specificity and increased retention of therapeutic doses for considerable improvement in the overall treatment of the tumors. In this case we describe here a synthetic approach to glycopolymer base nanoparticle gold(I) conjugate for cancer therapy.

Key words Glycopolymer, Gold(I) triphenylphosphine, Target specificity, Glyconanoparticles

1 Introduction

Cancer is a disease caused by aberrant cell cycle progression and defective apoptosis induction due to the activation of proto-oncogenes and/or inactivation of tumor suppressor genes [1]. Cancer is the leading cause of death worldwide, accounting for 8.2 million deaths in 2012 [2,3]. The burden is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices which include smoking, physical inactivity, and "westernized" diets [4]. It was estimated in 2012 that 14.1 million new cancer cases and 32.6 million people living with cancer (within 5 years of diagnosis) worldwide [5]. The current treatment of cancer involves radiation, chemotherapy, hormone ablation, and radical prostatectomy, which are associated with significant toxicity and recurrence risk [5,6]. To improve the outcome of patients with cancer, there is a need for new therapies, for higher cure rates while minimizing treatment-related toxicities. Advances in nanotechnology have led to the fabrication of nano-constructs of organic or inorganic origins with well-defined structures, surface properties, and can be made to respond to physical or chemical

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stimuli. These nano-constructs can provide a shift in the way diagnostic and therapeutic drugs are delivered to achieve target specificity and increased retention of therapeutic doses for considerable improvement in the overall treatment of the tumors. The evolving molecular events often provide the intervening candidate targets for the development of cancer therapy. Some of the most promising targets are ASGP receptors and p53, a well-established and frequently mutated tumor suppressor in human cancer. p53 for example plays a critical role in tumor suppression mainly by inducing growth arrest, apoptosis, and senescence, as well as by blocking angiogenesis. In addition, p53 generally confers the cancer cell sensitivity to chemo radiation. Thus, p53 and ASGP becomes the most appealing target for mechanism-driven anticancer drug discovery [7]. Therefore, our motivation is towards the development of novel glycopolymer nanomedicine for targeting ASGP receptors overexpressed on the liver cancer cellular surface. Glycopolymers and glyconanoparticles are of interest here as they expected to be safe nano-constructs for the safe delivery of the cancer drug ($AuPPh_3$). We have suggested three steps strategies to incorporate thiol and dithiocarbamate functionality for the stabilization of gold nanoparticles and the cancer drug for therapeutic application via RAFT polymerization method [8].

The first step is the fabrication of statistical glyco-glycidyldithiocarbamate polymers: p(GMA-EDAdtc-st-LAEMA) and p(GMA-EDAdtc-st-GAEMA) (GPdtc) (Fig. 1). This involves a three-step approach: (1) the statistical polymerization of gluconamidoethyl methacrylate and an epoxide (glycidyl methacrylate) (method section, subheading 1), (2) post-decoration of the epoxide with oligoamine to yield cationic copolymers P(GMA-EDA)-st-P(GAEMA) and P(GMA-EDA)-st-P(LAEMA) [9], and (3) the resultant cationic glycopolymer bearing terminal amino group contains primary amino groups as our functionalization targeted moiety to react with the activated carbon disulfide at 0 °C (Subheading 3) to give statistical glyco-glycidyldithiocarbamate polymers (Fig. 1).

In the second approach glyco-dithiocarbamate stabilized gold nanoparticles were synthesized via photoirradiation method using Irgacure 2959 as photoinitiator (Fig. 2). Finally, a spherical glyco-dithiocarbamate stabilized gold nanoparticles were conjugated to gold triphenylphosphine as described in Fig. 2.

2 Materials

2.1 Chemicals

1. 4-Cyanopentanoic acid dithiobenzoate (CTP).
2. Carbon disulfide.
3. Ethylene diamine (EDA).

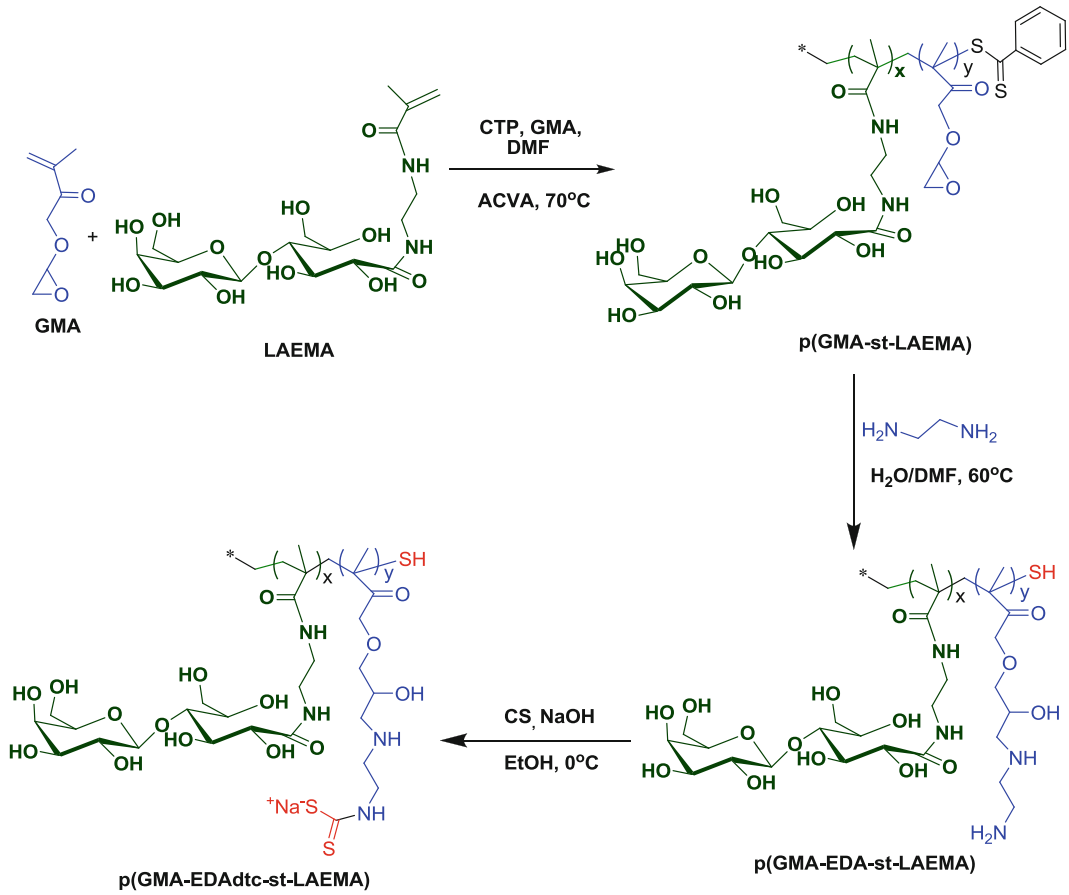

Fig. 1 Synthesis of statistical dithiocarbamate copolymer p(GMA-EDAdtc-st-LAEMA) (LPdtc)

4. Tetrahydrothiophene.
5. Hydrogen tetrachloroaurate.
6. Triphenyl phosphine.
7. Glycidyl methacrylate (GMA, 97 %, Aldrich) is purified by column chromatography using alumina prior to its use.
8. 4,4-Azobis (4-cyanovaleric acid) (ACVA) (Acros Organics).
9. 2-Gluconamidoethyl methacrylamide hydrochloride (GAEMA) and 2-lactobionamidoethyl methacrylamide (LAEMA) were synthesized as described in [10–13].

2.2 Buffer and Other Solutions

1. Doubly distilled deionized water is used in all experiments.
2. PBS buffer solution (pH 7.4) 1.0 M.
3. Buffer solution for GCP: sodium acetate 0.5 M/acetic acid.
4. Six near-monodisperse PEO standards (Mp) 1,010–101, 200 g/mol).

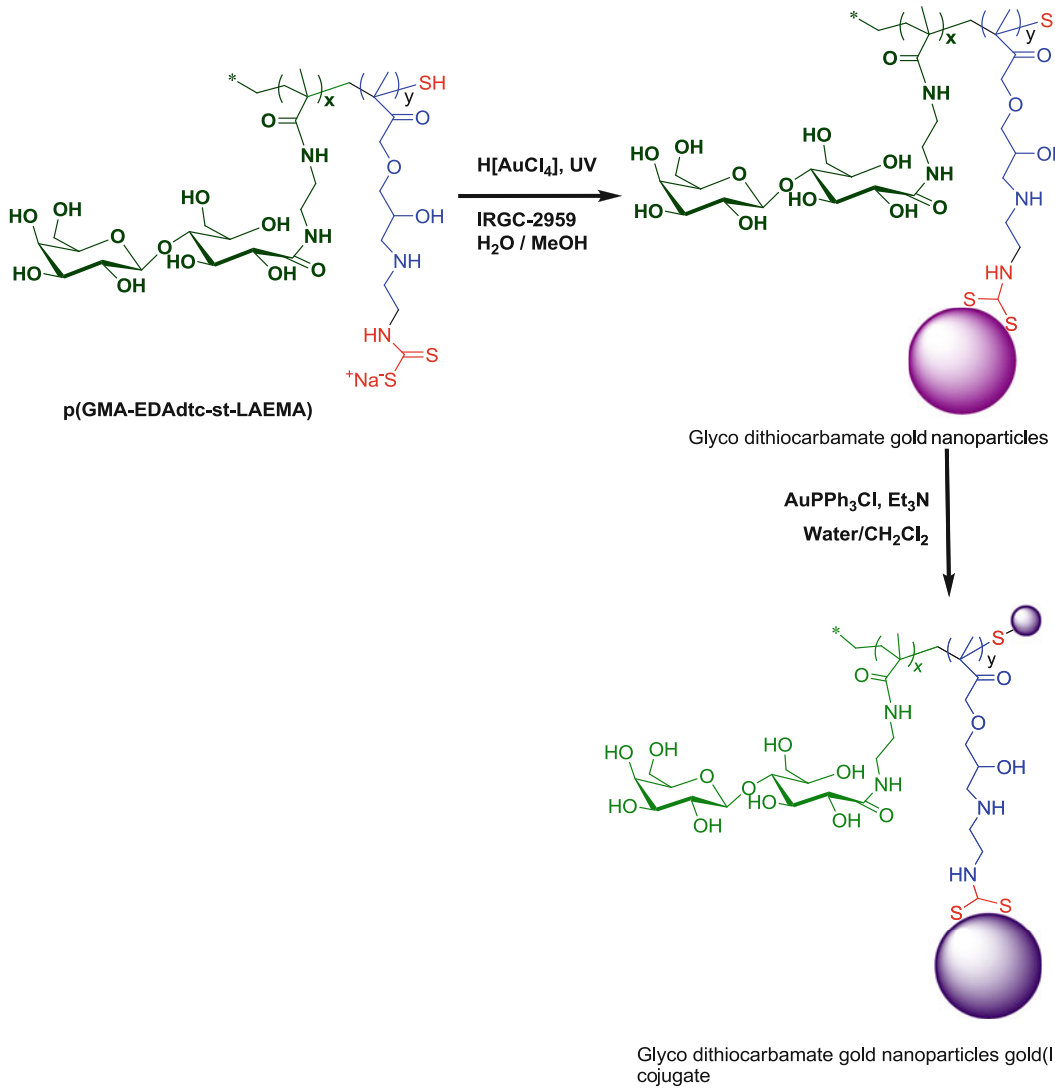

Fig. 2 Synthesis of polymer-functionalized gold nanoparticles and their conjugation to the anticancer drug, Au(1)PPh_3

5. 10 % NaOH (1.7 M) solution for critical flocculation concentration (CFC) measurement.

2.3 NMR Spectroscopy

^{1}H NMR spectra of the monomers and polymers are recorded using a Varian spectrometer (500 MHz).

2.4 Chromatography

2.4.1 Preparative GPC

Aqueous gel permeation chromatography analysis is performed on a conventional Viscotek Instrument using 0.5 M sodium acetate/0.5 M acetic acid buffer as eluent, two Waters WAT011545 columns at room temperature and a flow rate of 1.0 mL/min using 0.5 M sodium acetate/0.5 M acetic acid buffer as eluent.

2.4.2 Analytical GPC

1. Seven near-monodisperse Pullulan standards (M_w 5900–404,000 g/mol) are used for calibration.
2. The products are analyzed by an Agilent HPLC 1100 interfaced with an Electro-Spray Ionization Agilent Mass Spectrometer Model 6120 with a Chemstation data system LCMSD B.03.01.

2.5 Fourier Transform Infrared

Fourier transform infrared (FT-IR) spectral analyses using KBr pellets of the synthesized samples are carried out on a Nicolet 8700 (Thermo) instrument and the diffuse reflectance spectra are scanned over a range of 4000–400 cm^{-1} wave numbers.

2.6 Dynamic Light Scattering

1. Dynamic light scattering (DLS) measurements are performed with a ZetaPlus-Zeta Potential Analyzer (Brookhaven Instruments Corporation) at a scattering angle $\theta = 90°$.
2. p(GMA-EDA-st-LAEMA)-stabilized gold nanoparticles solutions are filtered through Millipore membranes (0.45 μm pore size).
3. The data are recorded with Omni size software.

2.7 UV–Visible Spectroscopy

UV–visible absorption spectra (400–800 nm) were recorded on a Cary UV 100 spectrophotometer from the aqueous solutions of polymeric AuNPs at room temperature.

2.8 Photoirradiation

The photo irradiation of the reaction mixture was carried out using 16, 12, 8, and 4 75 W UV lamps at a wavelength of 300 nm in a Rayonet photo reactor (Southern N.E. Ultraviolet Co.).

3 Methods

The RAFT polymerization approach and two-step strategies were used to prepare stable surface-functionalized gold(I) dithiocarbamate gold nanoparticles conjugate. Firstly, RAFT statistical copolymers of glycidyl methacrylamide (GMA) and 2-gluconamidoethyl methacrylamide, P(GMA-st-GAEMA), glycidyl methacrylamide, and 2-lactobionamidoethyl methacrylamide P(GMA-st-LAEMA) were synthesized employing ACVA as the initiator and CTP as the chain transfer agent, according to previously reported procedures (*see* **Note 1**) [8,14,15] as described in details below. This was followed by post-decoration of the glycidyl methacrylamide epoxide surface with ethylene diamine (EDA) to yield cationic copolymers: $P(GMA\text{-}EDA)_7$-st-$P(GAEMA)_{22}$ and $P(GMA\text{-}EDA)_{8\text{-st-}P}(LAEMA)_{24}$ with molar masses of *ca.* 20 and 22 kDa, respectively, with narrow molecular weight distributions ($M_w/M_n < 1.33$). In this step the terminal dithioester RAFT group was reduced to free thiol by

aminolysis during the amine decoration (Subheading 3) [16] (*see* **Note 2**). This thiol group was targeted as the stabilizing agent of the triphenylphosphine as the dithiocarbamate moiety was believed to stabilize the gold nanoparticles or vice versa. Lastly, the DTC polymers were synthesized by mixing EDA-decorated polymers and carbon disulfide (*see* **Note 3**) and were characterized by ^{1}H NMR and FTIR spectroscopy. The average hydrodynamic diameter of dithiocarbamate polymers in 150 mM PBS was determined by DLS. Average sizes of 27.0 and 44.7 nm with moderate PDI (~0.30) were recorded [17,18]. The net charge of polymeric dithiocarbamate derived from LAEMA and GAEMA were slightly negatively charged (−10.13 and −5.00), suggesting that all free amines in the polymers were functionalized with a dithiocarbamate motif.

The second step is the glyco-dithiocarbamate polymer-coated gold nanoparticles synthesis via photo irradiation using Irgacure-2959 (IRGC) as a photoinitiator (*see* **Note 4**) and was confirmed by transmission electron microscopy (TEM), UV–Vis spectroscopy, and DLS analysis.

The size distributions of glyco-dithiocarbamate polymer-coated gold nanoparticles were relatively narrow with polydispersity index (PDI) between 0.17 and 0.28 and the particle size distributions as measured by TEM and DLS for all the GNPs ranged between 12.5 ± 3.7 and 45.8 ± 12.7 nm (Fig. 5) [18]. The stability of glyco-dithiocarbamate polymer-modified gold nanoparticles was assessed by the dispersion of nanoparticles in high salt and pH conditions. No shift of the UV–Vis peak was observed before and after GNPs suspension in 10 % NaCl solution, even after 1 week at normal temperature an indication that GNPs are stable in physiological conditions (Fig. 3a). The gold nanoparticles were also found to be reasonably stable at physiological pH, as a bathochromic shift of ~3 nm was observed at pH values between 6 and 8, with no broadening of surface plasmon resonance (SPR) band (Fig. 3b) [18].

Finally, a biologically active glyco-decorated gold nanoparticles gold(I) conjugates were prepared using glucose-derived and galactose-decorated dithiocarbamate-stabilized gold nanoparticles of about 13 nm in size and gold(I) triphenylphosphine chloride in a biphasic medium (Fig. 2). The successful conjugation of gold(I) was followed by TEM, UV–visible spectroscopy, DLS, and physical appearance. The UV–Vis spectra showed a red shift of absorption band after the conjugation of gold(I) onto the surface of polymeric galactose gold nanoparticles suggesting an aggregation of particles after conjugation which was evidenced in the TEM micrograph (Fig. 5). This was also evidenced by the change of color from red to purple (Fig. 4) after conjugation. The glycopolymer functionalized gold(I) conjugate revealed an increase in size (e.g., 13.0–21.4 nm), which confirms the successful conjugation of the glycopolymer to the gold triphenyl phosphine (Fig. 5c, d) [18].

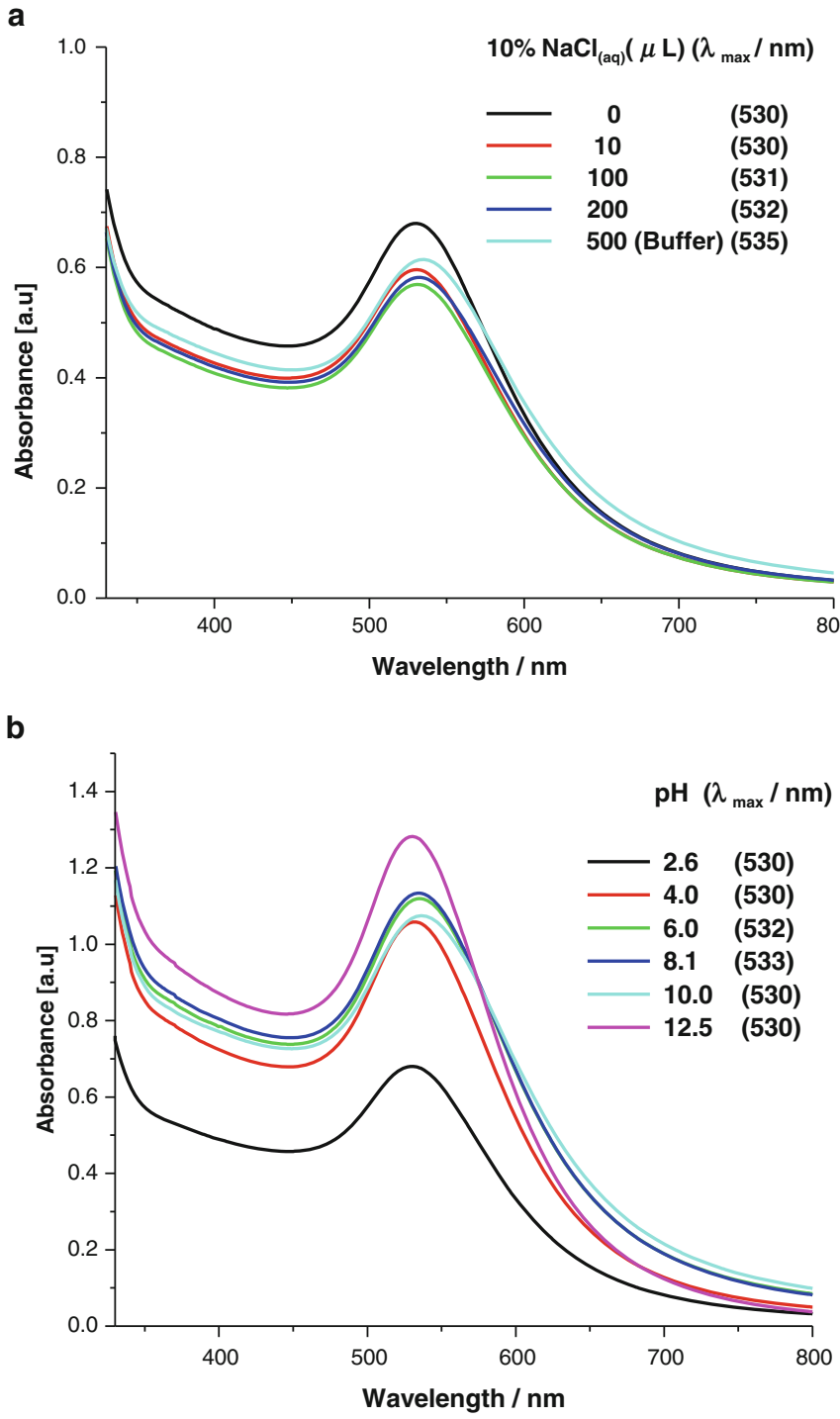

Fig. 3 Representative UV–Vis spectra of (**a**) gold nano particles after their dispersion in high-salt solution of 10 % NaCl and (**b**) gold nanoparticles at various pH using glyco-dithiocarbamate polymer-coated gold nanoparticles [P(GMA-EDAdtc-st-LAEMA)]AuNP [18]

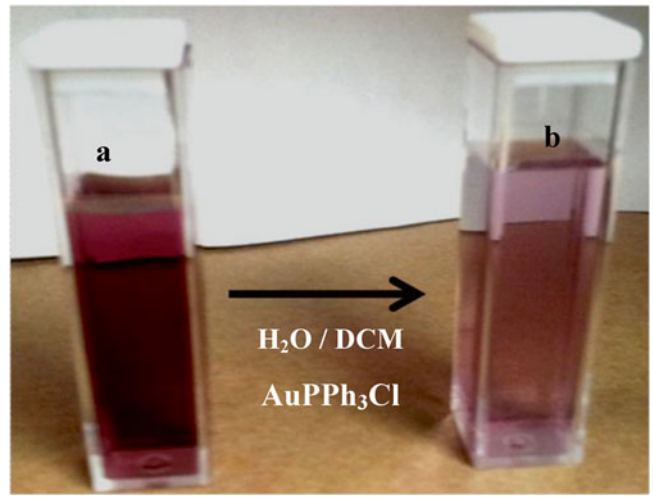

Fig. 4 Synthesis of galactose dithiocarbamate-coated gold nanoparticles gold(I) conjugate (**a**) EDAdtc-st-LAEMA)]AuNP, (**b**) [P(GMA-EDAdtc(Au(I)PPh_3)-st-LAEMA)]AuN [18]

3.1 Synthesis of Glyco-Decorated Dithiocarbamate Statistical Polymers

1. In a 10 mL flask, dissolve LAEMA (1.00 g, 2.04×10^{-3} mol) and GMA (0.17 g, 1.17×10^{-3} mol) in doubly distilled water (4.5 mL).
2. Add a solution of ACVA (3.5 mg, 0.0125 mmol) and 4-cyanopentanoic acid dithiobenzoate (CTP) (17.8 mg, 0.062 mmol) in N,N'-dimethylformamide (DMF) (1 mL).
3. Degas the mixture via three freeze-pump-thaw cycles for 30 min and seal with parafilm.
4. Place the reaction flask to a pre-heated oil bath at 80 °C and stir the mixture for 24 h.
5. Placed the reaction flask into liquid nitrogen to quench the polymerization.
6. Pour reaction mixture into diethyl ether precipitate out the polymer.
7. Dissolve the precipitated polymer into 5.0 mL of DMF and dialyze against 4 L double distilled water, replace three times per day over the course of 3 days (using pore size 12 kDa cutting molecular weight to remove unreacted monomer) and freeze dry to yield white solid.
8. Conduct the polymerization with a second monomer, GMA (0.166 g, 1.17×10^{-3} mol) and GAEMA (1.07 g, 3.5×10^{-3} mol) in water (5 ml), ACVA (3.5 mg, 0.0125 mmol), and 4-cyanopentanoic acid dithiobenzoate (CTP) (17.4 mg, 1.17 mmol) in 1 mL 1,4-dioxane also treated as steps above to yield second polymer as pink solid.

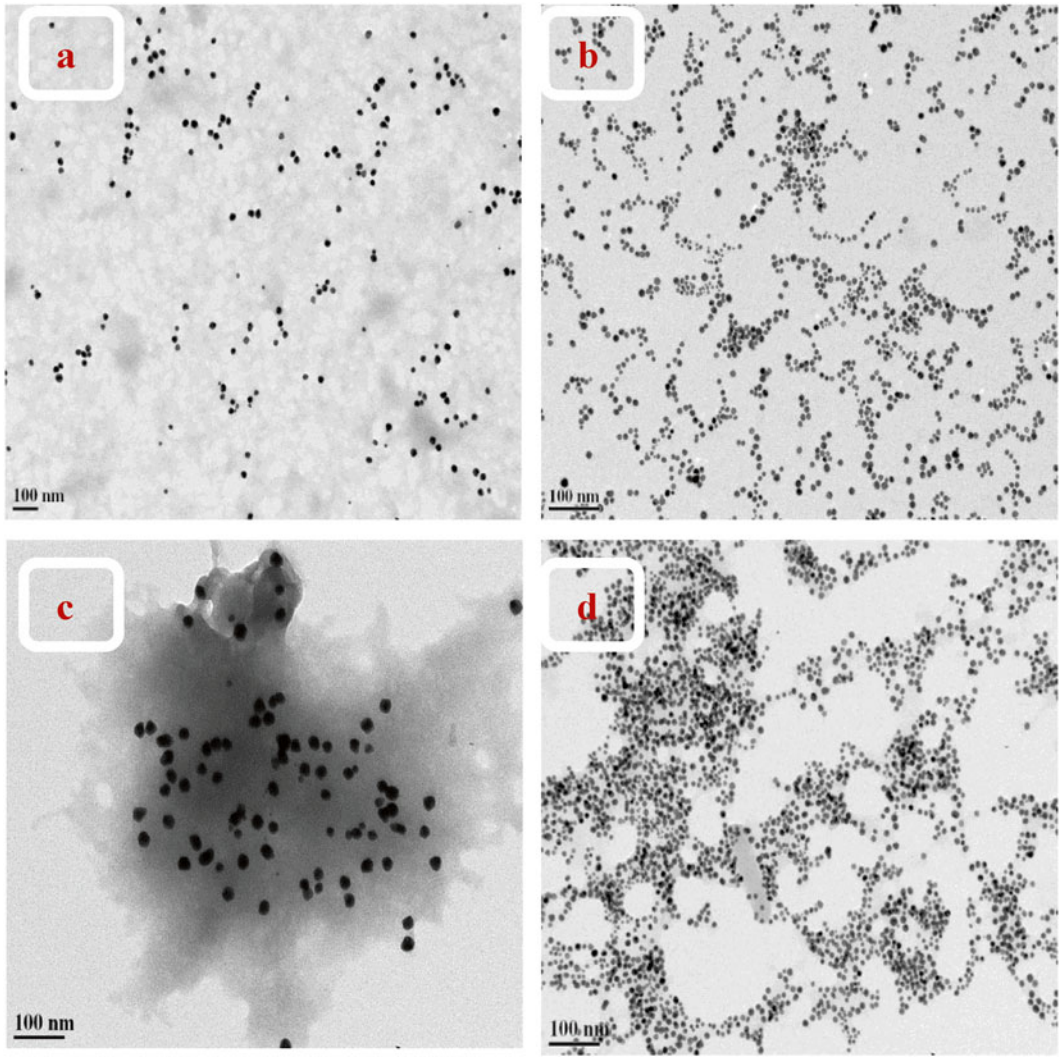

Fig. 5 Representative TEM micrograph of glyco-dithiocarbamate polymer-coated gold nanoparticles: (**a**) EDAdtc-st-LAEMA)]AuNP, (**b**) [P(GMA-EDAdtc-st-GAEMA)]AuNP and glyco-decorated gold nanoparticles gold(I) conjugates: (**c**) [P(GMA-EDAdtc(Au(I)PPh_3)-st-LAEMA)]AuNP, (**d**) [P(GMA-EDAdtc(Au(I)PPh_3)-st-GAEMA)]AuNP [18]

3.2 Decoration of Statistical Copolymers by Ethylenediamine (EDA) [p(GMA-EDA)-st-p(LAEMA)]

1. Dissolve EDA (2.0 mL, 10.5 mmol) in anhydrous DMF (10 mL) in a 25 mL round-bottom flask equipped with a magnetic stirring bar.
2. Add the respective polymers (50 mg) in 2 mL of DMF drop wise.
3. After reaction at 60 °C for 7 h, dialyze the reaction mixture (pore size 12 kDa cutting molecular weight) against 4 L of distilled water, exchange water three times per day over the course of 3 days.
4. Finally, freeze-dry to afford the amine decorated polymers for further characterization.

3.3 Synthesis of Polymeric DTC Polymers

1. Dissolve EDA decorated polymers (100 mg) in 0.25 M NaOH ethanolic solution (8.0 mL) and cool the solution to 0 °C.
2. Add carbon disulfide (0.04 mL, 0.71 mmol) in ethanol (2 mL) in drops wise.
3. Stir the reaction mixture for about 4 h at 0 °C and allow the mixture to stir overnight to afford the product.
4. Collect the solid by filtration and wash with ethanol to remove excess starting materials.
5. Dry the solid under vacuum to afford a degree of colored products, depending on monomer and molecular weight targeted. p(GMA-EDAdtc)-st-p(LAEMA) (LPdtc).

3.4 Synthesis of Statistical Glucose-Derived and Galactose Copolymer-Coated Au Nanoparticles in the Presence of Irgacure-2959 (IRGC)

1. Dissolve polymers: LPdtc (ca. 22 kDa) and GPdtc (ca. 20 kDa) in deionized water (15 mL) via sonication.
2. Add the solution to $HAuCl_4$ (0.005 mg/mL) solution in double-distilled water (5 mL).
3. Add a methanolic solution of Irgacure-2959 (8.5 mg, 0.038 mmol) initiator (5 mL) to $HAuCl_4$ and polymer mixture and degas via three freeze-pump-thaw cycles for 15 min
4. Conduction irradiation with photo reactor using sixteen, twelve, eight, and four 75 W UV lamps at wavelength 300 nm in a Rayonet photo reactor (Southern N.E. Ultraviolet Co.) for 15 min.
5. Take off the reaction mixture from the photo reactor and stir at room temperature for 5 min.
6. Collect the gold nanoparticles produced by filtering through Millipore membranes (0.45 μm pore size) and by centrifugation at 47,850 × *g* force for 2 h to remove excess polymer.
7. Re-suspend the pellet in deionized water and stored in a fridge for further characterization and use.
8. Three forms of polymer concentrations (1, 2, and 5 mg) gold nanoparticles are synthesized to study concentration variation effect.

3.5 Synthesis of Statistical Copolymer AuNP-Decorated Gold(I) Triphenylphosphine Complex

1. Add a solution of $[AuCl(PPh_3)]$ (2 mg, 0.004 mmol) and triethylamine (8.5 μL) in dichloromethane (5 mL) to a solution of respective polymeric modified galactose dithiocarbamate-stabilized AuNPs in water (6 mL; ca. 53.5 nM) under nitrogen.
2. Stir the resulting solution for 3 h at room temperature (23 °C).
3. Separate the aqueous layer and centrifuge at 47,850 × *g*-force for 2 h three times to remove unwanted materials
4. Resuspend the pellet in water for further characterization.

3.6 Critical Flocculation Concentration

1. Centrifuge the filtered glyco-stabilized gold nanoparticles at 26,916 × *g*-force for 90 min at room temperature (23 °C).
2. Resuspend the modified particles in 1.0 M phosphate buffer solution.
3. Centrifuge three times to ensure complete removal of free ligands.
4. After each 5 min, add 500 μL of 1.0 M phosphate buffer as well as 10, 100, and 200 μL of 10 % NaCl (1.7 M) solutions to each solution and allowed standing for 1 h.
5. The approximate glyco-stabilized gold nanoparticles concentration of *ca*. 53.4 nM (3 mL) is used for critical flocculation concentration (CFC) test.

4 Notes

1. The RAFT polymerization is achieved at 80 °C.
2. In the preparation of amine-decorated glycopolymer the ethyl diamine (EDA) is in a 30-fold molar excess and the reaction is carried out at 60 °C.
3. The functionalization of amine glycopolymers is done under 0 °C and all values are based on the amount of the P(GMA) in the polymer.
4. In the synthesis of glyco gold nanoparticles the molar ratios of $HAuCl_4$/IRGC in double-distilled water is 1:3 as polymer concentration is varied.

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