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# **SURFACE FUNCTIONALIZATION OF GOLD NANOPARTICLES USING HETERO-BIFUNCTIONAL POLY(ETHYLENE GLYCOL) SPACER FOR INTRACELLULAR TRACKING AND DELIVERY**

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## ABSTRACT

For development of surface functionalized gold nanoparticles as cellular probes and delivery agents, we have synthesized hetero-bifunctional poly(ethylene glycol) (PEG, MW 1,500) having a thiol group on one terminus and a reactive functional group on the other for use as a flexible spacer. Coumarin, a model fluorescent dye, was conjugated to one end of the PEG spacer and gold nanoparticles were modified with coumarin-PEG-thiol. Surface attachment of coumarin through the PEG spacer decreases the fluorescence quenching effect of gold nanoparticles. The results of cellular cytotoxicity and fluorescence confocal analyses showed that the PEG spacer modified nanoparticles were essentially non-toxic and could be efficiently internalized in the cells within one hour of incubation. Intracellular particle tracking using Keck 3-D Fusion Microscope System shows that the functionalized gold nanoparticles were rapidly internalized in the cells and localized in the peri-nuclear region. Using the PEG spacer, gold nano-platform can be conjugated with a variety of biologically-relevant ligands such as fluorescent dyes, antibodies, etc in order to target, probe, and induce stimulus at the target site.

## INTRODUCTION

Nanotechnology is expected to have a revolutionary impact on medicine. A variety of medical processes occur at nanometer length scales. Among the approaches for exploiting developments in nanotechnology in medicine, nanoparticles offer some unique advantages as sensing, delivery, and image enhancement agents [1,2]. Several varieties of nanoparticles are available [3]: polymeric nanoparticles, dendrimers, metal nanoparticles, quantum dots, liposomes, micelles, and other types of nano-assemblies. All of these nanostructures can play a major role in medicine, and especially in disease diagnosis and therapy.

With rapid advances in molecular biology and genetic engineering, there is an unprecedented opportunity for delivery of drugs and genes to intracellular targets [4]. In the case of cancer, for instance, the effectiveness of many anticancer drugs is limited due to the inability to reach the target site in sufficient concentrations and exert the pharmacological effect. Current intracellular delivery systems are classified as being either viral or non-viral in origin. Viruses are efficient in delivery; however, they suffer from poor safety profile [5,6]. Non-viral delivery systems, albeit not as efficient as viruses, have promise of safety and reproducibility in manufacturing [7]. To enhance delivery of drugs and genes to intracellular targets using non-viral delivery systems, it is necessary to have a detailed understanding of the transport process and identify ways of overcoming the cellular barriers [8].

Nanoparticles based on gold chemistry have attracted significant research and practical attention recently. They are versatile agents with a variety of biomedical applications including use in highly sensitive diagnostic assays [9], thermal ablation and radiotherapy enhancement [10,11], as well as drug and gene delivery [12]. For instance, antibody-modified gold nanoparticles when used for detection of prostate specific antigen, had an almost a million-fold higher sensitivity than conventional ELISA-based assay [13]. Near-infrared radiation absorbing gold-silica nanoshells have been prepared and evaluated for thermal ablation of tumors after systemic administration [14].

For biomedical applications, surface functionalization of gold nanoparticles is essential in order to target them to specific disease areas and allow them to selectively interact with cells or biomolecules. Surface conjugation of antibodies and other targeting moieties is usually achieved by adsorption of the ligand to the gold surface. Surface adsorption, however, can denature the

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proteins or, in some cases, limit the interactions of the ligand with the target on the cell surface due to steric hinderance. Additionally, for systemic applications, long-circulating nanoparticles are desired for passive targeting to tumors and inflammatory sites. Poly(ethylene glycol) (PEG)-modification of nanoparticles affords long circulating property by evading macrophage-mediated uptake and removal from the systemic circulation [15]. Surface modification of gold nanoparticles through the PEG spacer would, therefore, allow the modified nanoparticles to remain in the systemic circulation for the prolonged period and provide flexibility to the attached ligand for efficient interaction with its target.

In the present study, we describe the synthesis and characterization of hetero-bifunctional PEG with a thiol group on one terminus and a reactive functional group on the other for conjugation to a biologically-relevant targeting moiety. Using thiol-PEG-coumarin, a model fluorescent dye, we describe the “proof of concept” for surface functionalization of gold nanoparticle, cellular cytotoxicity evaluations, and fluorescence confocal analysis of cell uptake and nanoparticle distribution.

## EXPERIMENTAL PROCEDURES

**Synthesis and Characterization of Coumarin-PEG-Thiol:** A versatile hetero-bifunctional polyethylene glycol (PEG) compound containing end-groups thiol and alcohol derivatized as the coumarin carbamate was efficiently prepared as shown in Figure 2. The key step for the PEG synthesis involves the desymmetrization of the PEG-diol compound. This was achieved by monotosylation of the PEG-diol purchased from Aldrich (Milwaukee, WI) using the method of Bouzide and Sauve [16].

**Synthesis of Derivative 1:** To a chilled (0°C) solution of the PEG ( $M_w$ = 1427 g/mol, 12.0 g; 8.40 mmoles) in methylene chloride (distilled, 250 mL) was added freshly prepared  $Ag_2O$  (1.50 equiv., 2.92 g; 12.6 mmoles) and recrystallized potassium iodide (0.40 equiv., 558 mg; 3.36 mmoles). To this rapidly stirred solution was added in one-portion, purified tosyl chloride (TsCl) (1.05 equiv., 1.68 g; 8.82 mmoles). The reaction was deemed to be complete in 1-2 hrs by TLC (alumina). This was done by UV monitoring in the consumption of the tosyl chloride (TsCl) reagent and appearance of the tosylate product as a weakly UV active compound. The mixture was then carefully filtered over a pad of celite, and the clear filtrate obtained was then evaporated under reduced pressure to give a colorless oily product. Trituration with portions of ether to remove any trace TsCl reagent, followed by recrystallization (acetone/dry-ice bath) using methylene chloride-ether mixture provided the monotosyl-PEG derivative as a white amorphous solid. Yield: 12.5 g (95%);  $^1H$  NMR ( $CDCl_3$ , 300 MHz)  $\delta$ 7.80 (d,  $J$ = 8.1 Hz, Ar), 7.35 (d,  $J$ = 8.1 Hz, Ar), 4.16 (t,  $CH_2OTs$ ), 3.86-3.39 (m,  $29 \times CH_2$ ), 3.43 (t, 2H,  $CH_2$ ), 2.83 (t, 2H,  $CH_2$ ), 2.63 (br, s, OH), 2.45 (s, Me tosyl);  $^{13}C$  NMR ( $CDCl_3$ , MHz)  $\delta$ 143.95, 132.41, 129.10, 127.12, 125.4, 71.85, 69.74, 69.49, 68.60, 67.83, 60.66, 20.83 ( $MeSO_2$ ); IR ( $\nu$ ,  $cm^{-1}$ ) 3620-3192 (br, m), 2879 (br, vs), 1646, 1598, 1468, 1358 (asymmetric  $SO_2$  stretch), 1279, 1234, 1145 (symmetric  $SO_2$  stretch), 1112 (br, vs), 945, 927, 840; ESIMS calculated for  $MH^+ + H_2O$  (0.81)  $m/z$  1598.95, found 1598.91.

**Synthesis of Derivative 2:** A solution of the monotosyl-poly[ethylene glycol] (12.0 g; 7.59 mmoles), 10 equiv. of potassium thioacetate (8.67 g; 75.9 mmoles) in dry MeOH (300 mL) was heated to reflux for 48 hr under an atmosphere of nitrogen. The solution was allowed to cool to room temperature, and the MeOH removed using a Buchi rotary evaporator. The solid reaction mixture was treated with methylene chloride and the solution filtered. Concentration under

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reduced pressure gave the crude product, which was purified by chromatography using alumina and by eluting with 1% acetic acid/1% MeOH in methylene chloride. This purification yielded the product as a pale-yellow oil; and crystallization using ether (dry ice/acetone bath) provided the PEG thioacetate derivative as a pale-yellow solid. Yield: 10.1 g (90%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  4.18 (t, 2H,  $\text{CH}_2$ ), 3.94-3.62 (m, 58H,  $29 \times \text{CH}_2$ ), 3.41 (t, 2H,  $\text{CH}_2$ ), 2.83 (m, 2H,  $\text{CH}_2$ ), 2.81 (br s, 1H, OH), 2.04 (s, 3H,  $\text{SAC}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , MHz)  $\delta$  227.78 (SC[O]), 72.12, 70.05, 69.83, 69.21, 61.04, 28.33 (SC[O]Me); IR ( $\nu$ ,  $\text{cm}^{-1}$ ) 3603-3277 (br, m), 2888 (br, vs), 1697, 1466, 1359, 1347, 1277, 1242, 1112 (br, vs), 1061 (s, SO stretch), 948, 842; MALDI calculated for  $\text{M} + \text{Na}^+ + \text{H}_2\text{O}$  (1.67)  $m/z$  1538.90, found 1538.88.

**Synthesis of Derivative 3:** To a solution of the monothioacetate-poly[ethylene glycol] (2.50 g; 1.68 mmoles) in toluene (70 mL) was added freshly prepared coumarin isocyanate (1.05 equiv., 355 mg; 76 mmoles). The solution was heated to reflux for 12 hr under an atmosphere of nitrogen. Then, the mixture was allowed to cool and the toluene was removed using a buchi rotary evaporator. The crude product mixture was then treated with methylene chloride and the solution filtered over a pad of celite. Evaporation of the filtrate and then purification using alumina, by eluting with 1-2% MeOH in methylene chloride) gave the product as a pale yellow-colored oil. Finally, crystallization using ether (dry ice/acetone bath) provided the coumarin-PEG-thioacetate derivative as a pale-yellow solid. Yield: 2.41 g (85%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  7.87 (br s, 1H, NH), 7.53-7.41 (m, 3H, Ar), 6.17 (s, 1H, vinyl), 4.20 (t, 2H,  $\text{CH}_2$ ), 3.87-3.40 (m, 60H,  $30 \times \text{CH}_2$ ), 2.83 (m, 2H,  $\text{CH}_2$ ), 2.40 (s, 3H, vinyl Me), 2.08 (s, 3H,  $\text{SAC}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , MHz)  $\delta$  227.48, 160.15, 153.59, 152.55, 151.64, 141.82, 124.33, 114.07, 113.92, 111.81, 104.97, 71.83, 70.03, 69.71, 69.57, 68.50, 63.42, 60.71, 29.14 (SC[O]Me), 17.76 (Me); IR ( $\nu$ ,  $\text{cm}^{-1}$ ) 3558.04 (w), 2870 (s), 1732 (C=O, carbamate), 1694, 1620, 1575, 1533, 1463, 1350, 1291, 1231, 1108, 943; MALDI calcd, for  $\text{M} + \text{Na}^+ + \text{H}_2\text{O}$  (2.45)  $m/z$  1754.13, found 1754.15.

**Synthesis of Derivative 4:** A solution of the bifunctional coumarin-poly[ethylene glycol]-thioacetate compound (0.50 g; 0.30 mmoles) in degassed methanol (50 mL) was treated with 5 equivalents of degassed 0.5 M NaOMe/MeOH. The mixture was allowed to stir overnight at room temperature. Then, the mixture was acidified using DOWEX-DR 2030 resin to pH 1-2. The solution was quickly filtered over an overhead stream of nitrogen, and the solvent was removed using a rotary evaporator to give the crude product. Purification by silica gel chromatography and by eluting with 2-6% MeOH in methylene chloride gave the coumarin-PEG-thiol bifunctional derivative, which was obtained as a light-yellow solid upon further drying under diminished pressure. Yield: 375 mg (76%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  8.0 (br s, 1H, NH), 7.52-7.42 (m, 3H, Ar), 6.17 (s, 1H, vinyl), 4.35 (t, 2H,  $\text{CH}_2$ ), 3.94-3.63 (m, 58H,  $29 \times \text{CH}_2$ ), 3.50 (t, 2H,  $\text{CH}_2$ ), 2.83 (m, 2H,  $\text{CH}_2$ ), 2.41 (s, 3H, vinyl Me), 1.25 (br s, 1H, SH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , MHz)  $\delta$  227.48, 160.15, 153.62, 152.58, 151.64, 141.85, 124.34, 114.09, 113.96, 111.82, 104.99, 71.82, 70.05, 69.76, 69.55, 68.51, 63.43, 60.69, 29.53, 17.74; IR ( $\nu$ ,  $\text{cm}^{-1}$ ) 3421 (br, vs), 2918 (SH, m), 2882, 1651, 1463, 1351, 1297, 1251, 1100, 949; MALDI calculated for  $\text{M} + \text{Na}^+ + \text{H}_2\text{O}$  (3.99)  $m/z$  1739.84, found 1739.91.

**Preparation and Characterization of Coumarin-PEG-Functionalized Gold Nanoparticles:**

Gold nanoparticles were synthesized by reduction of gold chloride ( $\text{HAuCl}_4$ ) with freshly prepared sodium citrate and allowed to boil under reflux conditions. The pale yellow-colored solution turned to deep red as the gold nanoparticles were formed and stabilized by adsorbed chloride ions. Particle size analysis was performed with Coulter<sup>®</sup> N-4 sub-micron particle size analyzer (Coulter Corporation, Miami, FL). The nanoparticles suspension was air-dried on the specimen grid and observed with a transmission electron microscope (TEM)

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Coumarin-PEG-thiol was added to the aqueous dispersion of gold nanoparticle and the reaction for conjugation of the fluorescence dye to the gold surface through the thiol functionality was carried out at room temperature. The coumarin dye was used primarily as a demonstration of the covalent attachment, and confirms two important issues. First, because of the covalent bonding, the fluorescence observed is not from detached dye molecules, but from the fluorescent entity covalently attached to the gold nanoparticles. Second, the fluorescence is not quenched by the presence of the gold nanoparticles, probably due to the presence of the PEG spacer.

**Cytotoxicity Studies:** The *in vitro* cytotoxicity of the coumarin-PEG-thiol functionalized gold nanoparticles was measured using a commercially available CellTiter 96<sup>®</sup> AQueous Non-Radioactive Cell Proliferation Assay kit, purchased from Promega (Madison, WI). According to the manufacturer's instructions, the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, (MTS) is bio-reduced by viable cells into a formazan product. Approximately 10,000 MDA-MB-231 human breast carcinoma xenograft cells, suspended in Dulbecco's minimum essential medium (DMEM) modified with 10% (v/v) fetal bovine serum and other essential nutrients, were seeded per well into 96-well plates and allowed to adhere for 24 hours at 37°C and 5% CO<sub>2</sub> atmosphere. The medium was replaced with graded concentrations of polyethyleneimine (MW ~ 10,000) as a positive control; unfunctionalized gold nanoparticles and nanoparticles functionalized with methoxy-PEG-thiol (OMe-PEG-SH) as negative controls; and gold nanoparticles functionalized with coumarin-PEG-thiol as the test preparation in serum free medium (SFM) and incubated for 24 hours. An additional control wells received only SFM. After the incubation time, the cells were washed once with sterile phosphate buffered saline (PBS, pH 7.4), followed by addition of 180 µl of DMEM per well. The plates were incubated for 4 hours after the addition of 20 µl of MTS solution to each well and the absorbance of the formed formazan product was read at 490 nm using a microplate reader. The absorbance values were converted to percentage viability against control and reported as mean and standard deviation.

**Intracellular Trafficking Studies Using Keck 3-D Fusion Microscope System:** The Keck 3-D Fusion microscope system was developed at Northeastern University based on a commercially available Nikon TE2000U inverted microscope. It has five different modes of operation on one common platform: quadrature microscopy (QM); differential interference contrast (DIC); confocal fluorescence microscopy (CFM); 2-photon confocal laser scanning microscopy with second-harmonic generation (2PM); and confocal reflectance microscopy (CRM).

A known number of cells were grown on circular glass cover slips in a six-well plate containing DMEM. Once they had reached suitable confluency, the medium was replaced with SFM and the controls and test preparations were added. The final concentration of coumarin-PEG-thiol functionalized gold nanoparticles was 100 µg/ml per well and the samples were incubated for one hour. The cells were washed with sterile PBS twice and fixed with paraformaldehyde solution [0.5% (w/v) in PBS]. The cover slips were mounted (with cell side down) onto clean glass slides using Fluoromount G mounting medium. The fluorescent images were registered using an in-house built Keck 3-D Fusion microscope system using a 100x oil immersion objective.

We used the 2-photon laser scanning microscopy to measure the fluorescent signatures of coumarin-PEG-gold nanoparticles in the cells. The sample slide was placed on the stage with sample side down. The immersion oil was required for the objective. The tunable titanium-sapphire laser was set to be 780 nm for illumination. And the detection was done with a sensitive head-on photo multiplier tube (PMT) (HC1024-02, Hamamatsu).

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## RESULTS AND DISCUSSION

In the present study, we have synthesized hetero-bifunctional PEG having thiol group on one termini and an alcohol carbamate on the other for covalent attachment of coumarin. The PEG derivative was synthesized in high efficiency and characterized during each step of the synthesis pathway. Surface functionalization through PEG spacer would allow for flexibility in the attached molecule for more efficient interactions with the biological target. Otsuka, *et al.* [17] reported on the synthesis of hetero-bifunctional PEG synthesis having acetal-PEG-SH functionalities for attachment of lactose and other sugar molecules. They have also evaluated the interactions of the surface-attached sugar residues on gold nanoparticles with specific lectins for development of biosensors.

As shown by the Coulter particle size analysis results in Figure 2, we can reproducibly prepare 10-15 nm gold nanoparticles with a narrow size distribution. TEM image shows that the nanoparticles have a spherical shape. The surface of gold nanoparticles was functionalized with coumarin-PEG-thiol by covalent conjugation reaction. The unbound coumarin-PEG-thiol was removed by multiple centrifugation and washing steps and the emission from bound dye was measured with a fluorescence spectrophotometer. Although gold nanoparticles are known to be very efficient fluorescence quenchers [18], the attachment of coumarin through PEG spacer did show significant emission intensity. The PEG-modified gold nanoparticles (with methoxy-PEG-thiol and coumarin-PEG thiol) did not aggregate even in high ionic strength solution and confirm that the stable surface presence of PEG was effective in steric repulsion of the gold nanoparticles.

Cellular cytotoxicity studies in Figure 3 show that neither gold nanoparticles, gold nanoparticles functionalized with methoxy-PEG-thiol (i.e., gold-PEG-OMe nanoparticles) nor coumarin-PEG-thiol functionalized gold nanoparticles were cytotoxic at the concentrations studied. In contrast, polyethyleneimine, a known cytotoxic compound, did reduce cell viability to 40% of the control at the same concentrations.

As shown in Figure 4, upon incubation with MDA-MB-231 cells in culture, the coumarin-PEG-thiol functionalized gold nanoparticles were internalized by non-specific endocytosis within the first few minutes. Within 30 minutes, a large fraction of the administered dose was found to be in the early endosomes. As time progressed, the nanoparticles traversed through the cytosol and reached the perinuclear region within 1 hour of incubation. The hetero-bifunctional PEG derivative with a molecular weight 1,500 daltons was sufficient to allow flexibility in the attached fluorescent ligand, but did not prevent cellular entry of the nanoparticles. In addition, we did not observe any of the gold nanoparticles inside the nucleus even after 24 hours of incubation. Several recent studies, including some from our group [19], have shown that when nanoparticles are internalized in cells through endocytotic process, they tend to converge to the perinuclear area. Suh, *et al.* [20] attributed this directed intracellular movement of nanocarriers as an active transport process driven by molecular motor proteins. Using the Keck 3D Fusion microscope system, fast data acquisition, and custom particle tracking software, we can track functionalized gold nanoparticles inside the cells.

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## **CONCLUSIONS**

Gold nanoparticle surfaces were functionalized with coumarin, a fluorescent dye, through PEG spacer and used for intracellular tracking in NIH-3T3 murine fibroblast cells. Using the Keck 3-D Fusion microscope system, we can track functionalized nanoparticles with nanometer accuracy. The results show that these nanoparticles were rapidly internalized in the cells through non-specific endocytotic pathway and were found in the peri-nuclear region within one hour of incubation. Using the PEG spacer, the gold nano-platform can be conjugated with a variety of stimulus (e.g., cell penetrating peptides, endosomal buffering agents, etc) and probes (e.g., radiolabel, fluorescent dye, etc) moieties and used to study cellular transport pathways and barriers to efficient gene transfection.

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## FIGURE LEGENDS

- Figure 1.** Schematic illustration for the chemical synthesis of hetero-bifunctional poly(ethylene glycol) having thiol group on one terminus and coumarin carbamate on the other for attachment to gold nanoparticles (A) and surface functionalization of gold nanoparticles with coumarin-PEG-thiol (B).
- Figure 2.** Coulter particle size analysis (A) and transmission electron microscopy image of gold nanoparticles (B).
- Figure 3.** Cytotoxicity analysis of the controls and coumarin-poly(ethylene glycol)-thiol functionalized gold nanoparticles in MDA-MB-231 human breast adenocarcinoma cells. Bare gold nanoparticles were used as a negative control and polyethyleneimine (MW 10,000) was used as a positive control.
- Figure 4.** Cellular uptake and distribution of coumarin-poly(ethylene glycol)-thiol functionalized gold nanoparticles in MDA-MB-231 human breast adenocarcinoma cells. Differential interference contrast (A), epifluorescence (B), merged images (C), and 3-D plot of fluorescence intensity distribution as a functional of the X and Y coordinates was plotted (D). Original magnification was 100X.

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Figure 1.

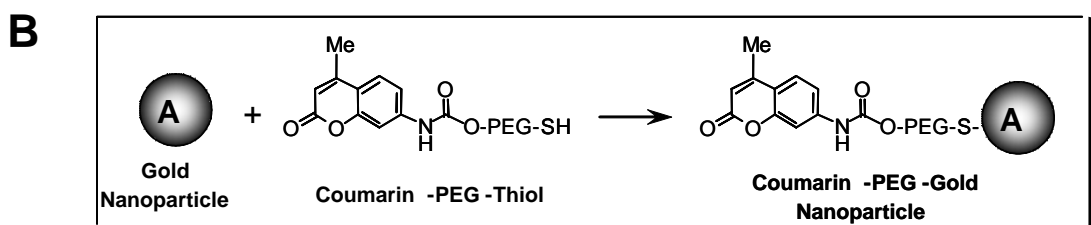
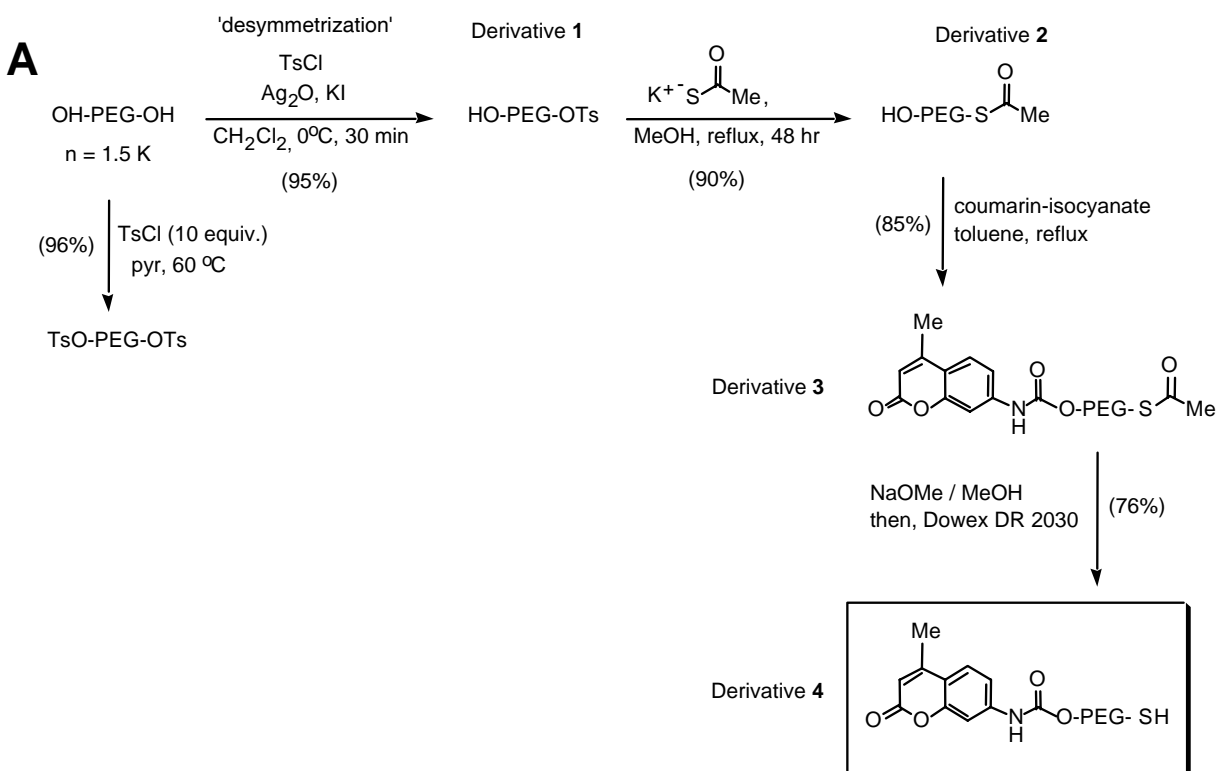


Figure 2.

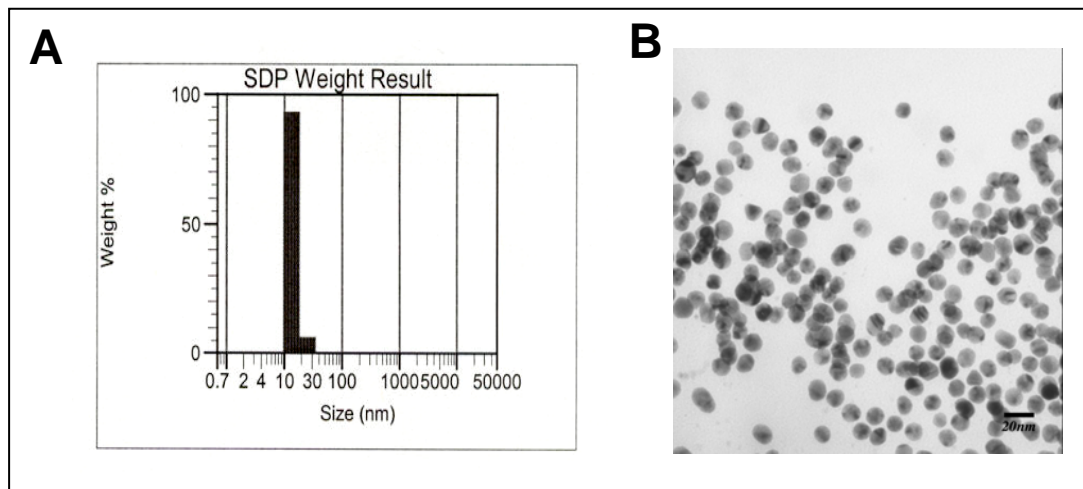


Figure 3.

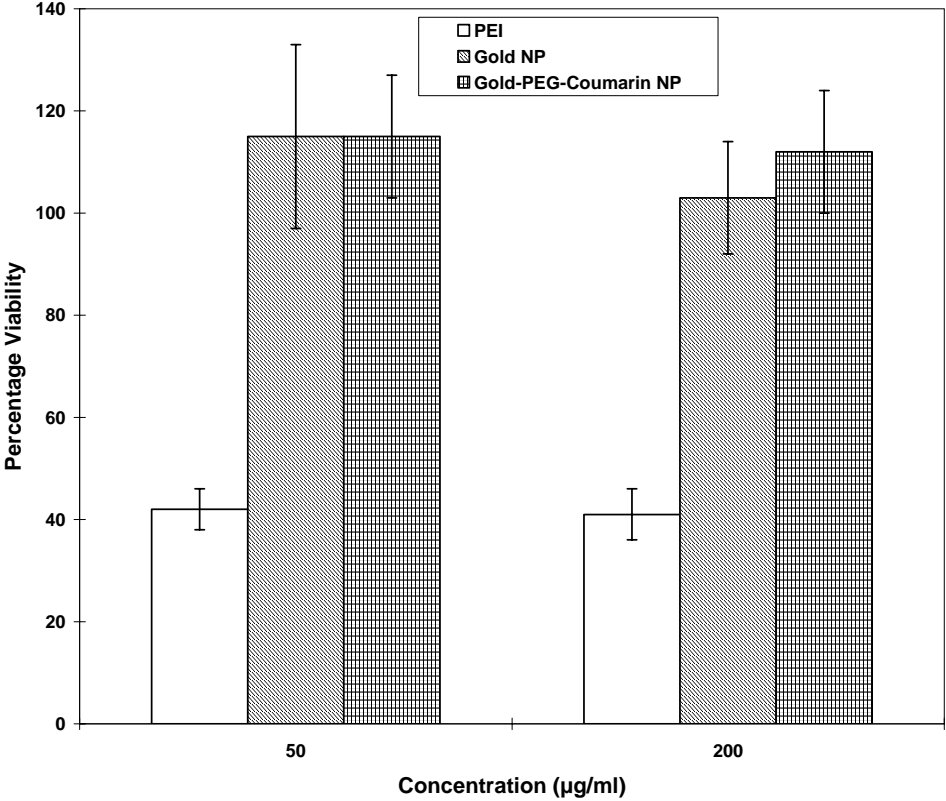


Figure 4.

