Highly Stable Positively Charged Dendron-Encapsulated Gold Nanoparticles

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ABSTRACT: We report the development of a novel cationic dendron (TAG1-PCD) and a positively charged gold nanoparticle–dendron conjugate (PCD-AuNP). TAG1-PCD was designed by considering the reactivity, hydrophilicity, and cationic nature that is required to yield a stable gold conjugate in aqueous media. The PCD-AuNPs, nominally 10 nm in size, were synthesized by reduction of chloroauroic acid in the presence of TAG1-PCD. The physicochemical properties of PCD-AuNPs were characterized by dynamic light scattering, transmission electron microscopy, UV–vis absorbance, and X-ray photoelectron spectroscopy for investigation of size distribution, shape uniformity, surface plasmon resonance bands, and Au-dendron bonding. Asymmetric-flow field flow fractionation was employed to confirm the in situ size, purity, and surface properties of the PCD-AuNPs. Additionally, the stability of PCD-AuNPs was systematically evaluated with respect to shelf life determination, stability in biological media and a wide range of pH values, chemical resistance against cyanide, redispersibility from lyophilized state, and stability at temperatures relevant to biological systems. Dose dependent cell viability was evaluated in vitro using the human lung epithelial cell line A549 and a monkey kidney Vero cell line. Observations from in vitro studies are discussed. Overall, the investigation confirmed the successful development of stable PCD-AuNPs with excellent stability in biologically relevant test media containing proteins and electrolytes, and with a shelf life exceeding 6 months. The excellent aqueous stability and apparent lack of toxicity for this conjugate enhances its potential use as a test material for investigating interactions between positively charged NPs and biocellular and biomolecular systems, or as a vehicle for drug delivery.

1. INTRODUCTION

Among the many classes of nanomaterials, gold nanoparticles (AuNPs) remain one of the most attractive nanoscale platforms for investigating biological interactions with nanomaterials and for development of nanomedicine applications. This interest is due in part to their general biocompatibility (which is not necessarily true for much smaller gold nanoclusters). AuNPs offer many advantages for biomedical applications, including facile conjugation with functional and bioactive ligands, including proteins and peptide chains, via thiol or amine bonding, and controllable particle size and shape; these and other attributes have been discussed at length in the literature. Utilization of AuNPs in biological applications includes diagnostics, drug carriers, sensors, and imaging. To further improve these applications, researchers have exploited the aforementioned conjugation chemistry to develop novel AuNPs to improve stability in physiological media, surface functionality, or to increase the structural complexity. In particular, positively charged gold nanoparticles (PC-AuNPs) are of interest in terms of cellular uptake, sensing capability through hybridization with biomolecules, transfection efficiency, and electrocatalytic behavior. These properties are related to the cationic nature of PC-AuNPs, which induce electrostatic interactions with negatively charged entities such as cell membranes, plasmid DNA, siRNA, or antibodies.

Interestingly, some studies have reported cytotoxic AuNPs capped with cationic ligands with toxicity a function of dose and/or exposure duration. Other studies have observed nontoxic effects of PC-AuNPs toward target cell lines. Due to the lack of control over NP agglomeration in test media and insufficient characterization of the agglomeration state before, during, and after interaction with cells and cell media, the mechanisms of toxicity and our understanding of how PC-AuNPs influence cellular processes remains ambiguous at best. We can state that the interaction of positively charged NPs with the surface of cell membranes suggests that PC-AuNPs could induce cellular uptake and/or toxicity, which can be either intentional (and therefore beneficial) or undesirable depending on the specific biological application. Paradoxically, this subtle

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aspect of PC-AuNPs could be used to advantage in order to afford a selective role as either drug delivery platform or active nanoscale positive control in toxicological studies. To date, there is a paucity of broadly validated nanoscale positive controls to conduct more accurate and reliable toxicity assays in vitro or in vivo, although NIST reference material AuNPs have shown promise as a nanoscale negative control for cytotoxicity studies.

A successful candidate NP for biomedical or toxicological applications must exhibit several universal attributes, including size monodispersity, long-term chemical and physical integrity, colloidal stability in physiological media and over the biologically relevant pH range, stability at physiological temperatures, and resistance to chemically induced degradation. Although there are many reported studies involving PC-AuNPs developed for a wide range of applications, physical and chemical stability are rarely investigated or reported, and this remains a considerable challenge (particularly in complex media, such as those used for cell culturing). Furthermore, in our experience, it is difficult to reproduce PC-AuNP syntheses from published studies where robustness of the NP fabrication procedure has not been systematically investigated. Repeated attempts in our laboratory to reproduce the materials reported in a number of these studies have yielded almost universally poor results (i.e., not consistent with reported properties) and nanoparticles with only transitory stability in suspension.

As part of our effort to investigate potential PC-NPs for use as positive controls, we have developed a novel and highly stable PC-AuNP obtained using dendritic architecture. We find that the cationic functionalized dendron and its respective AuNP conjugate yield excellent stability relative to other tested PC-AuNPs. Recently, we documented the development of an anionic dendron-stabilized AuNP, including synthesis, characterization, and stability evaluation under relevant conditions. Building on this approach, we have designed and synthesized dendron-encapsulated PC-AuNPs incorporating useful synthetic strategies previously reported by the Mattoussi and Rotello research groups. Positively charged dendron-encapsulated AuNPs, hereafter referred to as PCD-AuNPs, were prepared by reduction of HAuCl₄ in the presence of the

Scheme 1. Synthesis of Positively Charged Dendron (4) and Its Gold Nanoparticle Conjugate (PCD-AuNP)\(^\text{a}\)

\[\text{H AuCl}_4 + 4 \rightarrow \text{PCD-AuNPs}\]

\(^{a}\)(i) Dicyclohexylcarbodiimide, 1-hydroxy benzotriazole, PEG (M₉ ≈ 600 Da), dimethylformamide, room temperature (r.t.), overnight; (ii) methanesulfonyl chloride, triethylamine, methylene chloride, ice-bath to r.t., overnight; (iii) ethanolic trimethylamine, 35 °C, 2 d.; (iv) sodium borohydride, H₂O, r.t., 2 h.
purified dendron. The PCD-AuNPs were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM), UV–vis absorbance, and X-ray photoelectron spectroscopy (XPS), for investigation of size distribution, shape uniformity, surface plasmon resonance (SPR) behavior, and chemical bonding (i.e., S–H bonding with Au), respectively. Also, asymmetric-flow field flow fractionation (A4F) was employed to confirm the in situ size, purity, and surface properties of PCD-AuNPs. We also conducted a systematic stability evaluation of the PCD-AuNPs, including long-term shelf life, in physiological media (e.g., phosphate buffered saline (PBS), and Dulbecco’s modifi ed Eagle’s medium (DMEM)), as a function of pH and temperature, chemical resistance against cyanide attack, and the lyophilization–reconstitution cycle.

2. EXPERIMENTAL SECTION

Specific reagents used in this study are identifi ed in the Supporting Information (SI). All chemicals were used without further purifi cation. Details regarding instrumentation and methodology are also provided in the SI.

2.1. Preparation of Dendrons and Their Au Conjugates.

**Thioctic-tri-(PEG[600]−Ms)**

To the stirred solution of TAG1-COOH (0.65 g, 1.5 mmol) in DMF (30 mL), N,N’-dicyclohexylcarbodiimide (1.02 g, 4.95 mmol), and 1-hydroxy-1H-benzotriazole hydrate (666 mg, 4.95 mmol) were added, and after 1 h, reaction mixture was added dropwise to a 50 mL solution of PEG in DMF (30 g, 50 mmol). The mixture was stirred overnight at 25 °C, after which a white precipitate was removed by filtration and the solvent was evaporated in vacuo to yield a gel; the gel was dissolved in CH2Cl2 and washed sequentially with saturated sodium bicarbonate, water, and brine, and then dried over anhydrous Na2SO4. The organic layer was concentrated in vacuo, and the crude material was purifi ed by column chromatography through silica-gel (CH2Cl2:MeOH 8:1, v/v) and followed by dialysis against deionized water (MWCO = 500–1000, cellulose ester membrane) for 2 days to yield (35%) 2 as a pale yellow oil. Final yield was 1.15 g.

**Thioctic-tri-(PEG[600]-NMe3)**

Thioctic-tri-(PEG-[600]-Ms) 3. Thioctic-tri-PEG[600] 2 (1.0 g, 0.46 mmol) was dissolved in 30 mL of CH2Cl2 and cooled in an ice bath. Triethylamine (1 mL) and methanesulfonyl chloride (0.5 mL) were added to a solution at 0 °C then stirred overnight at ambient temperature under N2. The reaction mixture was diluted with methylene chloride and washed with 2% HCl, saturated sodium bicarbonate, and water, followed by drying over anhydrous Na2SO4. The organic layer was concentrated in vacuo, and the crude material was purifi ed by column chromatography through silica-gel (CH2Cl2:MeOH 10:1, v/v) to give (86%) 3 as a pale yellow oil. Final yield was 0.95 g.

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500–1000, cellulose ester membrane) for 2 days to give (80%) 4 as yellow gel. Final yield was 0.69 g.

**AuNP–Dendron Conjugate (PCD-AuNP).** To an aqueous solution of HAuCl₄ (10 mL, 2.5 mmol/L), 1 mL of aqueous dendron TAG1-PCD (2.5 mmol/L) and 1 mL of freshly prepared NaBH₄ (50 mmol/L in H₂O) were added sequentially at room temperature. The color of the reaction mixture changed from pale yellow to deep ruby red immediately after addition was completed. After stirring for 2 h, the crude colloidal gold solution was dialyzed against DI water (MWCO = 10K, cellulose ester membrane) for 2 days to give (80%) 4 as yellow gel. Final yield was 0.69 g.

**Results and Discussion**

### 3.1 Synthesis of Dendron and Gold Nanoparticle–Dendron Conjugate

As summarized in Scheme 1, TAG1-COOH, a generation-1 dendron with a thioctic acid (TA) terminus and three terminal carboxylic acids, introduced in our previous work, is used as the starting point for the synthetic route. TAG1-COOH is first treated with excess polyethylene glycol (PEG, Mₐ 600 Da) to obtain the 1→3 PEGylated adduct 2, which is followed by mesylation with methanesulfonyl chloride to produce precursor 3. For the preparation of the targeted TAG1-PCD 4, thioctic-tri-(PEG₆₀₀)-Ms 3 is treated with ethanolic trimethylamine to yield a pale yellow oil. The resulting 1→3 branched cationic dendritic ligand (hereafter TAG1-PCD) is composed of a TA moiety, PEG chains, and quaternary ammonium terminal groups to provide reactivity, hydrophility/aqueous stability, and pH-independent cationic sites.

The PCD-AuNPs were prepared from HAuCl₄ with TAG1-PCD in the presence of sodium borohydride (NaBH₄) as a reducing agent (Scheme 1) to yield a translucent solution with a deep ruby red color. This product was purified by dialysis against deionized water. Further details of the synthetic procedures and structural analysis are described in the Experimental Section and the SI.

### 3.2 Characterization of PCD-AuNPs

The physicochemical properties of PCD-AuNPs were determined by a combination of complementary and orthogonal measurement techniques including DLS, UV–vis absorbance, TEM, XPS, and A4F. The z-average diameter of the initially purified PCD-AuNPs, obtained by DLS, was (16.3 ± 0.5) nm with a monomodal size distribution (polydispersity index = 0.173, Figure 1a). The calculated hydrodynamic size distribution gave no indication of significant agglomeration between the particles. The measured zeta potential was (+13.7 ± 0.7) mV at pH 5.9, which confirmed a positively charged corona surrounding the gold core. The uncertainty of z-average diameter and zeta potential represent the mean and one standard deviation of at least three measurements under repeatability conditions.

UV–vis absorbance measurements reveal an SPR band with a maximum wavelength (λmax) near 515 nm (Figure 1b) for the initially purified PCD-AuNPs, a typical value for AuNPs in this size range. TEM (example shown in Figure 1c), conducted within a month after synthesis and purification, yields a mean diameter of (7.5 ± 2.1) nm for the PCD-AuNP gold core, and indicates a spherical uniformity in particle shape. As expected, the TEM diameter of the PCD-AuNPs is smaller than the diameter obtained by DLS due to differences between number- and z-averages, and due to the presence of the dendron corona that contributes to the hydrodynamic envelope of the particles but is transparent to TEM. As a back of the envelope estimate, the TEM number average can be converted to a z-average size using the relationship d_z = d_n / (polydispersity index) and due to the presence of the dendron corona that contributes to the hydrodynamic envelope of the particles but is transparent to TEM. As a back of the envelope estimate, the TEM number average can be converted to a z-average size using the relationship d_z = d_n / (polydispersity index) and due to the presence of the dendron corona that contributes to the hydrodynamic envelope of the particles but is transparent to TEM. As a back of the envelope estimate, the TEM number average can be converted to a z-average size using the relationship d_z = d_n / (polydispersity index). The size difference can be attributed to the 600 Da PEG spacers inserted into the structure of this ligand (TAG1-PCD), between the thioctic acid and the terminal quaternary ammonium moieties. Although only a rough estimate, the results are consistent with expectations.

The PCD-AuNPs were also analyzed by XPS (conducted after repeated purification steps) to identify the local chemical environment of the Au and S atoms and to confirm the presence of Au–S bonds. Figure 1d presents the spectra for Au (4f) and S (2p) regions; S (2p) and Au (4f) regions and...
components were adjusted with elemental relative sensitivity factors (RSFs) of 0.668 and 6.25, respectively. The Au (4f) spectra consists of two clearly separated peaks at 83.8 eV (4f7/2) and 87.5 eV (4f5/2), which can be assigned to metallic Au. The dominant spectral feature in the S (2p) region is at 161.1 eV (2p3/2) feature), comparable with previously observed measurements of thiolate species.46–48 This suggests that the main contribution to the S (2p) spectra is S bound to Au. In addition to the thiolate functionality, there was also residual, unattached dendron in the PCD-AuNP samples, as evidenced by the well-separated sulfonate peak at approximately 167.5 eV (S 2p3/2) and the disulfide peak at 163.5 eV (S 2p1/2), consistent with XPS measurements of the dendron alone (SI Figure S1). Last, a fourth functionality was fit to the S (2p) region at approximately 161.9 eV (S 2p1/2). This may represent residual S species formed by cleaving the five-membered ring of the thiotic acid moiety on the TAG1-PCD, but which is either not chemically bonded to Au or is involved in monodentate bonding. The measured peak intensity ratio, \( \frac{S_{\text{thiolate}}}{Au} \) (4f7/2)/Au (4f7/2), was 0.069 ± 0.005 (mean with one standard deviation of three separate, RSF-adjusted peak height measurements). It is important to note that this S/Au ratio can be used as a metric for relative surface coverage (RSC) allowing deviations of three separate, RSF-adjusted peak height measurements.

Figure 3 visual observation of PCD-AuNPs obtained by (a) reduction of HAuCl₄ in the presence of PCD, and (b) ligand exchange reaction with 10 nm citrate-stabilized AuNPs. Red color is indicative of a stable AuNP suspension, while purple indicates aggregation leading to a shift in the characteristic SPR band. (c) TEM image of citrate-AuNPs (nominal 10 nm), and (d) product from ligand exchange reaction (solution b).

3.3. Ligand Exchange Reaction. For comparison, we used direct ligand exchange of the TAG1-PCD with citrate-stabilized 10 nm AuNPs; this procedure was successfully used in our previous study involving carboxylated dendrons.15 Briefly, 1 mL of aqueous dendron TAG1-PCD (2.5 mmol/L) was added dropwise to 10 mL of citrate-stabilized AuNPs (nominally 10 nm) at room temperature. In the present case, the reaction mixture quickly changed color from red to purple and became turbid; within 1 h, the particles began to precipitate (compare Figure 3a and b). TEM imaging (Figure 3d) of sample deposited from this suspension 15 min after ligand exchange confirmed the altered colloidal state. It is worth noting that aqueous phase surface modification of citrate-stabilized AuNPs with a wide range of cationic ligands, including cystamine, cysteamine, polyethyleneimine (PEI, \( M_r \approx 5 \text{ kDa} \)), CTAB, and amino-PEG-thiol (\( M_r \approx 5 \text{ kDa} \)), each exhibited similarly poor results with particles quickly precipitating or otherwise exhibiting incipient destabilization (unpublished results). This phenomenon can be attributed to molecular bridging between citrate coated surfaces mediated by the cationic TAG1-PCD and other cationic ligands, as previously reported in the literature,51 which does not occur when place exchange is performed using like charged species.

3.4. Evaluation of Stability. Physical and chemical stability are critical issues for any commercial application of colloidal AuNPs. We evaluated the stability of the PCD-AuNPs over a range of relevant conditions utilizing previously established protocols.15 Native PCD-AuNPs aged for 6 months under ambient laboratory conditions yielded a size distribution (from DLS) and UV−vis absorbance spectrum (Figure 1a,b) that were essentially identical to the freshly prepared and purified product. Additionally, zeta potential measured for the initial and aged samples (data not shown) was effectively equivalent (within typical measurement uncertainty), evidence for a largely stable surface chemistry. These results suggest that there is little change in the physicochemical properties with respect to at least a 6-month shelf life under ambient laboratory storage conditions.

\[ S_{\text{thiolate}} (2p3/2)/Au (4f7/2) = 0.082 \]

Visual observation of the synthesized AuNP conjugates,47 and is not an atomic or molar ratio but an XPS intensity ratio. The RSC for S/Au bonding was calculated by normalizing the relative sensitivity factor for PCD-AuNPs to that for mercaptoundecanoic acid conjugated AuNPs (MUA-AuNPs), \( S_{\text{thiolate}} (2p3/2)/Au (4f7/2) = 0.082 \), with the assumption that the latter peak intensity ratio represents maximal surface density for a thiol ligand on AuNPs. The RSC for the PCD-AuNPs was 0.83 ± 0.06, or 83% relative to MUA-Au.15

A4F is an in situ separation technique that we have effectively used in a previous study to fractionate and characterize citrate-stabilized AuNPs.49 An A4F fractogram captures the retention behavior of the analyte over time using optical absorbance or scattering detectors. The elution of negatively charged \( 10 \text{ nm citrate-AuNPs} \) (Figure 2a, solid line) yields a typical light scattering (LS) trace, while online DLS (Figure 2a, circles) confirms the size. When PCD-AuNPs (Figure 2a, dotted arrow) are analyzed using the same conditions, the baseline scattering indicates that the particles are completely deposited from this suspension 15 min after ligand exchange and aged samples (data not shown) was essentially identical to the freshly prepared and purified product. Additionally, zeta potential measured for the initial and aged samples (data not shown) was effectively equivalent (within typical measurement uncertainty), evidence for a largely stable surface chemistry. These results suggest that there is little change in the physicochemical properties with respect to at least a 6-month shelf life under ambient laboratory storage conditions.
Figure 4. Stability of PCD-AuNPs in biological test media over 48 h, as monitored by UV−vis absorbance and z-average diameter from DLS (inset): (a) PBS, (b) DMEM.

Figure 5. Stability of PCD-AuNPs as a function of pH over a 12 h period (a, b) and decomposition rate in aqueous KCN solution (c): (a) pH 3, (b) 50 mmol/L NaOH (pH 12.7), (c) PCD-AuNPs (open circles), Au-TAG1-COOH (open triangles), and 10 nm diameter citrate-stabilized AuNPs (closed circles) in 2 mmol/L KCN shown as normalized absorbance at 520 nm.
Stability in physiologically relevant aqueous media was tested over a 48 h period relevant to many cell-based exposure assays. PCD-AuNPs diluted into PBS exhibited excellent stability based on hydrodynamic size and absorbance spectra (Figure 4a). We attribute this behavior to the hydrophilicity of the PEG chain and dendritic steric repulsive interactions that substantially reduce salt sensitivity (screening of electrostatic charge). Another common biological test medium that is often used as the basis for cell culture medium is DMEM. The results for DMEM (Figure 4b) are more complex. The NP size as determined by DLS is unchanged over 48 h, but the SPR band intensity is reduced (without red-shifting) by about 20% over the same period. Normally, the reduction in the SPR absorbance would be attributed to removal of material, perhaps by agglomeration followed by rapid sedimentation. This cannot be ruled out here, but the constant DLS size and absence of red-shifting in the SPR band suggests that perhaps there is an incipient reaction between the PCD-AuNPs and the DMEM components that leads to the loss of signal. Overall, the PCD-AuNPs demonstrate a substantial improvement in stability relative to citrate-stabilized AuNPs and the other ligand-exchanged positively charged formulations tested in our laboratory (e.g., PEI 2 kDa, 10 kDa), which resulted in rapid (seconds to a few hours) precipitation under identical conditions.

The pH-dependent stability of PCD-AuNPs was evaluated over a 12 h period. Clearly, pH stability is another important consideration in the preparation of NPs for biomedical applications. The range of pH values potentially encountered in physiological systems is very broad. For example, the stomach can approach pH 1 due to secretion of hydrochloric acid, and lysosomes, which engulf and digest small intracellular objects like food or viruses, contain enzymes that are active at mildly acidic pH (∼4.8). Blood and intracellular fluids typically maintain a near-neutral pH, in the range 7.2 to 7.4, while the small intestine becomes increasingly basic as one moves from the upper to lower region, eventually reaching a pH of about 8. Notably, PCD-AuNPs exhibit very stable behavior down to at least pH 3 (Figure 5a), as the quaternary ammonium cationic groups associated with PCD-AuNPs, unlike lower order amines, are not susceptible to protonation. Furthermore, in strong acid (50 mmol/L HCl, pH ≈ 1.3), even though the PCD-AuNPs exhibit gradually decreasing absorbance and a slight red shift (Δλ ≈ 8 nm; SI Figure S2a) over 12 h, overall, the resistance against acid destabilization is greatly improved relative to citrate AuNPs and anionic dendron stabilized AuNPs, which gradually precipitate or significantly red-shift their SPR band when subjected to pH 3 (and precipitate immediately in strong acid). The improvement in the PCD-AuNP stability under acidic conditions can be attributed in part to the quaternary amines and possibly in part to the aliphatic chain that acts as a diffusion barrier against intrusion of acidified water to the AuNP core, which would likely result in aggregation and core fusion leading to precipitation. At pH 7.2 (SI Figure S2b) the PCD-AuNPs are stable, as expected; surprisingly, they are nearly as stable as at very alkaline pH conditions (50 mmol/L NaOH, pH ≈ 12.7) as shown in Figure 5b. It could be explained that quaternary ammonium functional groups on PCD-AuNPs do not form H-bonds between particles, and this absence of H-bonding provides stability in basic pH unlike the destabilization of amine terminated AuNPs induced by interparticle H-bonding at high pHs as reported by Lin et al. The chemical stability of the PCD-AuNP conjugates was evaluated by examining the NP resistance to cyanide etching. Rapid digestion of citrate-stabilized AuNPs by cyanide ions is well documented, and several studies have demonstrated retarded decay rates resulting from surface modifications. PCD-AuNPs and 10-nm-diameter citrate-stabilized AuNPs (for comparison) were rapidly diluted into aqueous KCN (2 mmol/L), and the decay rate was measured by monitoring the optical absorbance at 520 nm over a 6 h period. PCD-AuNPs exhibit a dramatically enhanced resistance (slower decay) relative to the citrate-capped particles (Figure 5c). The stability/surface modification relationship is well documented in our previous work. Briefly, chemical resistance is proportional to the S/Au intensity ratio (sulfur coverage effect) and diffusion barrier (branch effect) produced by the organic corona (ligand chain length or bulkiness, hydro-
phobicity). The PCD-AuNP corona contains both aliphatic chains and hydrophilic PEG chains, to provide hydrophobic and steric hindrance, and the PCD is anchored, as in our previous study of carboxylic terminated dendrons (TAG1-COOH), by two S–Au bonds resulting from the 1,2-dithiolane moiety. Notably, the resistance of PCD-AuNPs is almost identical to the result of the anionic dendron gold conjugates (Au-TAG1-COOH, S/Au = 0.93 ± 0.03)\textsuperscript{15} based on the similar decay rates. This similarity could be explained by the counterbalance between the sulfur coverage and branch effect for PCD-AuNPs and Au-TAG1-COOH, i.e., smaller RSC (0.83 ± 0.06), but more hydrophobic and longer chain length relative to Au-TAG1-COOH conjugates.

Thermal stability of PCD-AuNPs was evaluated by DLS and UV–vis absorbance measurements over the range from (20 to 60) °C, which covers the relevant range for most biomedical applications or biological assays. Citrate-stabilized AuNPs are generally insensitive to temperature over this range (data not shown), and we anticipated similar behavior for the PCD-AuNPs. Samples were incubated for 30 min at each temperature before measurements were conducted. The constancy of the z-average size (from DLS) and the SPR band (from UV–vis spectra) confirm that the PCD-AuNPs are stable with respect to temperature variations over the tested range (SI Figure S3).

Although the long-term colloidal and chemical stability of PCD-AuNPs has been demonstrated by monitoring of particle size and UV–vis absorbance spectra over a six month period (Figure 1a,b), it is well documented that the semicovalent S–Au bonds are subject to cleavage as a result of sulfur oxidation in air.\textsuperscript{58} More recently, evidence has suggested that cleavage of thiol-bonded ligands on AuNPs in solution can occur under typical laboratory storage conditions.\textsuperscript{59} The evidence also suggests that this cleavage may not be immediately reflected in the absorbance spectra. One possible route to improving long-term stability and retarding oxidative desorption of ligands is to lyophilize the NP suspension and store under inert atmosphere (e.g., under argon or nitrogen). To investigate this possibility, we assessed the response of PCD-AuNPs to a lyophilization–reconstitution cycle (absent the inert storage condition). It has been shown previously that citrate-stabilized AuNPs cannot be reconstituted following lyophilization,\textsuperscript{15,60} but AuNPs with other surface modifications have proven to have more successful reconstitution capacity, including NPs coated with ethylene–propylene oxide block copolymers\textsuperscript{60} and lipid bilayer protected AuNPs.\textsuperscript{61} As shown in Figure 6, analysis of reconstituted PCD-AuNPs lyophilized (without the aid of excipients) revealed excellent recovery of the primary NP size, with a 5 nm shift in the z-average value. Evidence for minor aggregation after reconstitution was indicated in the DLS-derived size distribution. On the other hand, there was no observable change in the SPR band \( \lambda_{\text{max}} \) at 515 nm following reconstitution. These results suggest that the majority of the PCD-AuNPs retain their pre-lyophilization singly dispersed state. This is fairly remarkable when compared to the citrate-capped AuNPs and other dendron stabilized AuNPs, which when subjected to the same process result in substantial agglomeration.\textsuperscript{15,16} Presumably, the improved stability is the result of the additional 1–3 PEG chains in the PCD. Further improvements in reconstitution may be achieved by judicious use of excipients commonly utilized as cryo- and lyoprotectants (e.g., polyols, sugars), a subject of ongoing research.

### 3.5. Evaluating the Effect of PCD-AuNPs on Biological Cells

Positively charged NPs have been shown to be both toxic and nontoxic in cell-culture based assays.\textsuperscript{31−34} To determine if the PCD-AuNPs induce a cytotoxic response in biological cells, we tested its effect in cell culture media using two different cell-culture systems. Media used for culturing cells in these experiments contain 10% (v/v) fetal bovine serum (FBS) proteins which could induce agglomeration and precipitation of the NP. Control experiments showed that the dispersion of PCD-AuNPs (40 µg/mL, the highest dose used for cytotoxicity studies) in 10% FBS in DMEM at 37 °C and 5% CO\textsubscript{2} environment (pH \( \approx 7.4 \)) did not cause changes in the S14
nm absorption signal over a 48 h period (SI Figure S4). The ruby red color of the dispersion could be clearly observed after 48 h suggesting that the PCD-AuNP remains unaggregated in the protein-containing cell culture media. Notably, 48 h exposure of African monkey kidney cells (Vero) and human lung carcinoma (A549) cells to increasing concentrations of PCD-AuNPs did not cause detectable decreases in cell viability as monitored with the MTS assay (Figure 7). Interestingly, a dose-dependent increase in MTS absorbance was detected in the Vero cell experiments. This may indicate an increase in reductase activity within the cells, possibly due to stress, or absorbance artifacts due to the absorption of PCD-AuNPs to the cell-covered well surface. Visual inspection of the Vero cultures with phase microscopy suggested that there was no obvious difference in the cell density or morphology for the treatment and nontreatment cell cultures, but the presence of large opaque structures were observed (SI Figure S5). A qualitative decrease in the ruby red color of the NP-treated media exposed to cells was also observed suggesting these structures are agglomerates of the PCD-AuNPs. The structures responded to flow currents from gentle pipetting action consistent with the structures being located on the external surface of the cells (data not shown). Since the PCD-AuNPs were found to be stable in the cell culture media without the presence of cells, these results suggest that the presence of the Vero cells are required to form the agglomerate structures (i.e., the effect is cell mediated). The presence of these structures on the cell surfaces may be responsible for the apparent dose-dependent increase in MTS absorbance observed in Figure 7. Further studies are currently underway to understand the formation and composition of these extracellular structures. Overall, these results suggest that the 40 μg/mL PCD-AuNP treatment did not induce a significant toxic response in the two cell lines tested here, but there is visible attachment of the PCD-AuNPs to the Vero cell membrane surface in a manner that leads to dense associations of particles, a phenomenon worth further investigation due to its implications for biological interactions of positively charged NPs.

4. CONCLUSIONS

The PCD-AuNP reported herein is a candidate for a nanoscale test material for cellular assays, or for use as a drug carrier in nanotherapeutics. In summary, we have successfully created a novel 1→3 directional first generation cationic dendron (TAG1-PCD), composed of a thiocetic acid moiety, three polyethylene glycol (PEG) chains (Mₙ ≈ 600), and three quaternary ammonium end groups. To obtain the stable conjugate, chloroauric acid was reduced in the presence of TAG1-PCD and the reducing agent NaBH₄, resulting in a positively charged dendron-conjugated AuNPs (PCD-AuNPs). The physicochemical properties of the PCD-AuNPs were fully characterized by complementary and orthogonal methods. The chemical and colloidal stability of the PCD-AuNPs were determined under physiologically relevant conditions, and proved superior relative to other conjugated AuNPs we have investigated and therefore suitable for consideration in applications requiring long shelf life. Several studies suggest that positively charged nanomaterials can elicit a response in cytotoxicity assays. When the material was tested on two cell lines from different species, a cytotoxic response was not observed at the concentrations used. Further studies will be required to determine if the particles are taken up by the cells by pinocytosis or other mechanisms or if the particles interact only with the cell membrane surface. The apparent attachment and agglomeration of the PCD-AuNPs induced by the presence of Vero cells suggests a biological origin for this phenomenon, as the NPs were stable in the cell culture matrix under identical conditions. Further research is planned to investigate this unusual and unexpected effect. Presumably, the NPs are also associated with protein components in the matrix (i.e., form a protein corona), but the solution retains a ruby red color indicative of little or no agglomeration of the particles, providing evidence that this material may be a biologically applicable template.


(18) Thomas, K. G.; Camat, P. V. Chromophore-Functionalized Au Nanoparticles and Their Use in Counting Surface Ligands. Small 2010, 6, 1273–1278.


