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Introduction of nanoscale positive controls for nanotoxicology: A new aspect of utilization of stability-enhanced Gold-Polyethyleneimine conjugates (*Au-PEIs*)

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***Abstract*— We report on a stability-enhanced suspension of gold-polyethyleneimine conjugates (Au-PEIs), prepared by an optimized synthetic process. The physicochemical properties of Au-PEIs were characterized using several analytical instruments including dynamic light scattering, UV-Vis, transmission electron microscope, and atomic force microscope. The nanoparticles in this report have shown excellent colloidal stability to avoid particle-particle interactions in various aqueous environments, which is very critical to become effective materials in nanomedicine. Most importantly, the cytotoxicity of Au-PEIs has been investigated by cell viability assays using a variety of cell lines (CHO K1, HeLa, and A549) and has significant toxicity in common, appeared to be more toxic than a traditional positive control ($CdSO_4$). This suggests the potential for use as a universal nanoscale positive control material in nanotoxicity studies.**

Keywords*— gold nanoparticles, polyethyleneimine, colloidal stability, nanotoxicity, nanoscale positive control

I. INTRODUCTION

Gold nanoparticles (AuNPs) are promising platforms for biomedical applications of nanomaterials, in part due to their general biocompatibility.[1, 2] Gold-Polyethyleneimine (PEI) conjugates (Au-PEIs) have potential use as positively charged gold nanoparticles (AuNPs) in nanomedical applications, due to their cationic surface that promotes cellular uptake and gene transfection. Polyethyleneimine (PEI) is one of the most studied cationic polyelectrolyte for its unique features such as hydrophilicity, complexation with metal ions[3] and polyanions,[4] and, most interestingly, gene transfection efficiency for innovative biological applications.[5] Therefore, by combination of AuNPs and PEI, Au-PEI conjugate is expected to generate synergistic effects leading to improved efficiency and efficacy in nanomedical applications due to their bio-compatibility (Au) and active interaction with biological entities (PEI), respectively. However, this active interaction between particles and bio-entity could lead to an undesirable effect, cytotoxicity. Paradoxically, this delicate feature of Au-PEI could be advantageous in providing a selective role as a drug delivery platform or active nanoscale positive control in the nanotoxicology field. In our best knowledge, there are no validated nanoscale positive controls for establishing standardized protocols of nanotoxicology assays,[6] meanwhile, AuNPs reference material (RMs 8011, 8012, and 8013, National Institute of Standards and Technology (NIST), Gaithersburg MD) have served as nanoscale negative controls for nanotoxicology.[7] In general, for NPs to be successful in biomedical or toxicological applications, they must exhibit several critical properties, including monodisperse size distribution, shape uniformity, optical property, long-term shelf-life, and colloidal stability in physiologically relevant conditions.[8] Previously, Cho et. al at NIST reported[9, 10] the synthesis of Au-PEI by a systematically investigated reduction method to elucidate the optimal conditions for producing stable, high quality Au-PEI, and therein, multi-parametric analysis of key factors was conducted. Herein, we introduce a very recently developed Au-PEIs (hereafter Au-PEI@NIST),[11] through the scale-up reaction modified from a previously optimized method,[9, 10] which exhibit high-quality physicochemical properties and outstanding colloidal stability as characterized by several orthogonal analyses. Most importantly, the cytotoxicity of this validated material, Au-PEI@NIST has been investigated using various cell lines, including CHO K1, HeLa, and A549

cells and showed substantial dose proportional toxicity in common, which suggests a potential to become a universal nanoscale positive control material in nanotoxicology.

II. Experiments[ξ]

A. Materials and Instruments

Gold[III] chloride hydrate ($HAuCl_4 \cdot 3H_2O$, ACS reagent) and branched 25 kDa PEI (bPEI25 kDa) were obtained from Sigma-Aldrich (St. Louis, MO). Methoxy polyethylene glycol thiol (OMe-PEG-SH; 5 kDa) was obtained from Laysan Bio Inc. (Arab, AL). Other specific reagents used in this study are described in [11]. Details regarding analytical instruments and methodology for characterization, stability, and toxicity tests are also provided in the literature.[9-11] The uncertainty of size and zeta potential (ZP) values by dynamic light scattering (DLS) represented by one standard deviation from at least three measurements. For transmission electron microscope (TEM) and atomic force microscope (AFM) measurements, more than 150 and 1300 particles were analyzed, respectively, and each size reported is the population mean with one standard deviation about the mean.

B. Preparation of AuNPs

1) Au-PEI@NIST

10 mL of aqueous bPEI25 kDa (10 % mass fraction) was added to 100 mL of aqueous $HAuCl_4$ (2.5 mmol/L) at room temperature (r.t.) and heated up to 80 °C using an oil bath with stirring. After reaching 80 °C, the reaction mixture was then stirred for additional 3 h. The oil bath was then removed to allow the reaction mixture cool down to r.t. and purified using a stirred cell ultrafiltration (Amicon, regenerated cellulose (RC) membrane, MWCO 100 kDa). In this step, the reaction medium is reduced from 100 mL to (10 to 20) mL, then backfilled with DI water to about 100 mL. This purification cycle is repeated 4 times with the final volume of Au-PEI@NIST suspension adjusted to ≈ 100 mL with DI water yielding an Au concentration of ≈ 2.5 mmol/L, which is similar to the starting concentration.

2) Au-PEG@NIST

100 mL of aqueous $HAuCl_4$ (0.25 mmol/L) was heated to a reflux. To this reflux, 1 mL of aqueous sodium citrate (120 mmol/L) was added and stirred for 30 min. Cooled the red suspension down to r.t., then added 1 mL of aqueous OMe-PEG-SH (5 mmol/L; molar mass 5 kDa) and stirred additional 3 h at r.t. The reaction mixture was purified by centrifugal filtration (Amicon ultra15, RC membrane, MWCO 100 kDa). *Note*: The concentration was adjusted to be similar as that of Au-PEI@NIST by reducing the final volume down to 10 mL (≈ 2.5 mmol/L of Au).

C. Cytotoxicity

Cytotoxicity of Au-PEI@NIST was evaluated for CHO K1, HeLa, and A549 cell lines using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell viability assay. The MTS assay is a screening assay and provides a rapid method to assess potential toxic interactions with biological cells. The reagent is reduced in the presence of cellular enzymes and forms a colored product that is soluble in the culture media. The optical density of the culture media measured at 490 nm follows cell count in the culture conditions. Measurement procedure was adopted from ISO 19007:2018; www.iso.org/standard/63698 (Nanotechnologies–*In vitro* MTS assay for measuring the cytotoxic effect of nanoparticles). In this study, a series dilution of Au-PEI@NIST are loaded in three replicates along with a negative control (Au-PEG@NIST), and a traditional positive control–cadmium sulfate ($CdSO_4$) also cerium oxide (CeO_2), as another type of cationic nanoparticles for comparison purposes.

III. Results and Discussion

A. Physicochemical properties

The physicochemical properties of Au-PEI@NIST were characterized by orthogonal measurement techniques including DLS for z-average hydrodynamic diameter (D_z), ZP for surface charge property, TEM for core diameter (D_{TEM}), AFM for particle height (H_{AFM}), and ultraviolet-visible spectroscopy (UV-Vis) for surface plasmon resonance (SPR) band. Representative measurements data of Au-PEI@NIST were presented in Fig. 1 and TABLE 1.

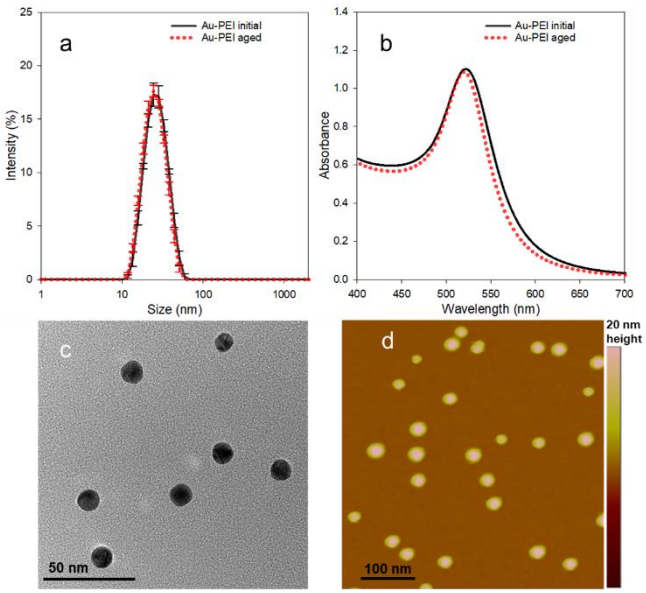

Fig. 1. Representative measurements data of Au-PEI@NIST; (a) intensity-weighted size distribution by DLS, (b) SPR band by UV-Vis, (c) TEM image (Scale bar is 50 nm), and (d) AFM image (Z-scale for height is 20 nm and scale bar for width is 100 nm; *Note*: The apparent width of the particles are larger than their height due to the intrinsic tip convolution effects in AFM imaging).

TABLE I. Fundamental properties of initial Au-PEI@NISTs

AuNPs @NIST	D_z (nm)	D_{TEM} (nm)	H_{AFM} (nm)	ZP (mV)	SPR (nm)
Au-PEI	24.2±0.2	11.8±1.8	10.4±0.2	+15.6±0.9	521
Au-PEG	38.4±0.6	N/A	N/A	-19.4±2.8	521

[ξ] The identification of any commercial product or trade name does not imply endorsement or recommendation by the National Institute of Standards and Technology.

As shown in TABLE I, the z-average diameter (D_z = (24.2 ± 0.2) nm) by DLS appears larger than other sizes obtained from TEM (D_{TEM} = (11.8 ± 1.8) nm) and AFM (H_{AFM} = (10.4 ± 0.2) nm). Hydrodynamic size of the AuNPs, including the gold core and surface ligand, is generally larger than the gold core size for a given particle. This is attributed to the difference of measurement techniques depend on instruments. For size determination, DLS is a method for diluted/suspended samples and TEM and AFM are methods for deposited/dried samples. The former offers a spherical-equivalent hydrodynamic size of the entire particles, and the latter allows visualization and measurements of the metal core size and shape of the tested particles. ZP reflects the surface charge properties of the suspended particles in a specific medium and the ZP of Au-PEI@NIST was (+15.6 ± 0.9) mV at pH ≈ 9, which indicates the cationic surface resulting from the PEI conjugation. The maximum absorption (λ_{max}) of the SPR band for Au-PEI@NIST appeared at 521 nm and this value is typical for this size range of spherical AuNPs. For comparison purposes in this study, Au-PEG@NIST was also prepared as a nanoscale negative control and showed corresponding properties such as D_z = (38.4 ± 0.6) nm, ZP = (-19.4 ± 2.8) mV , and λ_{max} = 521 nm for the maximum SPR band. No further size characterization by TEM or AFM for Au-PEG@NIST was necessary for this study.

B. *Colloidal Stability*

Colloidal stability is a critical issue for any biomedical application of AuNPs. We evaluated the stability of the Au-PEIs over a range of relevant conditions utilizing previously established protocols.[12]

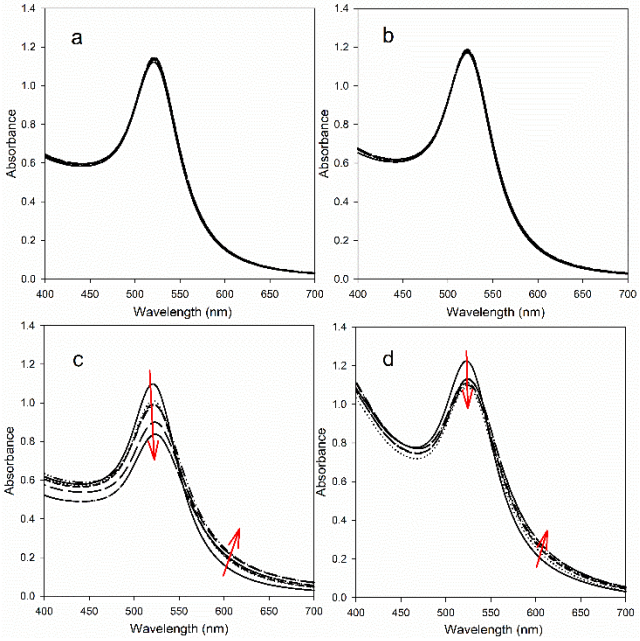

Fig. 2. Representative data showing the colloidal stability of Au-PEI@NIST over 72 h at 20 °C evaluated in physiological media (f = 10): by UV-Vis (a) PBS, (b) M9 buffer, (c) DMEM, (d) DMEM containing 10 % BSA.

Au-PEI@NIST yielded very similar size distribution and SPR band, before and after aging (Fig. 1a, b; black line for initial and red line for 3 year aged sample), without any detectable morphological change induced by particle-particle interaction (agglomeration or aggregation) that confirms an excellent long-term storage stability at r.t. without any excipients. Fig. 2 shows the colloidal behaviors of Au-PEI@NIST in physiological media. The SPR bands of Au-PEI@NIST exhibited identical spectral shape over 72 h in PBS (Fig. 2a) and M9 (Fig. 2b) buffer, respectively. Au-PEI@NIST obviously well tolerates charge screening and sustains its stable behavior although PBS and M9 buffer have sufficiently high ionic strength to yield strong charge screening effect that could lead to agglomeration of charge-stabilized particles due to their strong salinity composition. Dulbecco's Modified Eagle's Medium (DMEM), a common cell culture medium that includes biological ingredients in addition to salts, is a more complex medium. Au-PEI@NIST exhibited a ≈ 18 % decrease in the SPR maximum intensity and a slight red-shift ($\Delta\lambda_{max}$ = 2 nm) in UV-Vis spectra over 72 h in DMEM (Fig. 2c). These results can be interpreted that some of the non-salt components interact sufficiently with particles and induce a assessable agglomeration.[10] In addition, stability test in [DMEM + 10 % Bovine serum albumin (BSA)] (Fig. 2d) showed that the presence of a moderate concentration of serum protein can markedly recover stability. The SPR band was reduced by ≈ 8 % with red-shift ($\Delta\lambda_{max}$ = 2 nm) over the first 6 h, different from the DMEM only, where no further red-shift observed over 24 h. The initial red-shift could be attributed to conjugation between Au-PEI@NIST and BSA, there was no more morphological change apparent with test material for 72 h. In other words, there is an initial modest effect, then the complex showed decent colloidally stability for long periods of time in [DMEM + BSA]. Relevant supplemental study by DLS has been described elsewhere.[11] Over a wide range of pH values (pH 1.5 ~ 12), Au-PEI@NIST showed remarkable stability over 12 h (data omitted) demonstrating great resistance against acid destabilization. Thermal stability (tested from 20 °C to 70 °C) evaluated by monitoring UV-Vis spectra, showed invariable SPR band (from UV-Vis spectra, data omitted) and confirmed that the Au-PEI@NIST nanoparticles are stable within the tested temperature range.

C. *Cytotoxicity*

Cell viability is calculated from the MTS absorbance as follows: viability (%) = [(Experimental A_{490nm} - Background A_{490nm})/(Negative control A_{490nm} - Background A_{490nm})] x 100. The data are expressed as means ± one standard deviation from three measurements. The materials were incubated with cells for 24 h and reflects the acute response of the cells (threshold was not determined for *in vitro* case). Representative data in Fig. 3 shows that Au-PEI@NIST decreased cell viability in a dose dependent manner. The lethal dose for 50 % of CHO K1 and HeLa cells (representing normal cells and cancer cells, respectively) following 24 h estimate; LD_{50} = (12.2 ± 3.1) µmol/L and (8.4 ± 1.2) µmol/L, respectively (in Fig. 3, black bars), was obtained by approximating the viability data to a continuous concentration-response curve by a non-linear fit. These LD_{50} values indicate that the particle toxicity of Au-PEI@NIST to the tested mammalian cells was significantly higher than that of $CdSO_4$, the potent chemical toxin often used as positive control (pink bars, LD_{50} = (315.0 ± 10.1) µmol/L and (468.5 ± 36.0) µmol/L for CHO K1 and HeLa cells, respectively).

Moreover, a highly cationic CeO_2 NPs (d < 25 nm, average zeta potential in the growth media = (150.7 ± 21.4) mV) did not exhibit meaningful toxicity to CHO K1 and HeLa cells at concentrations up to 500 µmol/L (red bars, Fig. 3a, b). In addition, Au-PEG@NIST, the neutral AuNPs, showed markedly lower toxicity to both cell lines (LD_{50} = (184.0 ± 12.0) µmol/L and (330.5 ± 60.0) µmol/L for CHO K1 and HeLa cells, respectively) than positively charged Au-PEI@NIST. Furthermore, another type of cancer cell, A549 also showed significant toxic response to Au-PEI@NIST (LD_{50} = (16.0 ± 2.3) µmol/L, data not shown). Again, due to the stability of Au-PEI@NIST in test media, no significant visual (color) or morphological changes were observed before and after MTS assay. Overall, toxicology studies on Au-PEI@NIST demonstrate significant toxic effects in common to tested cell lines.

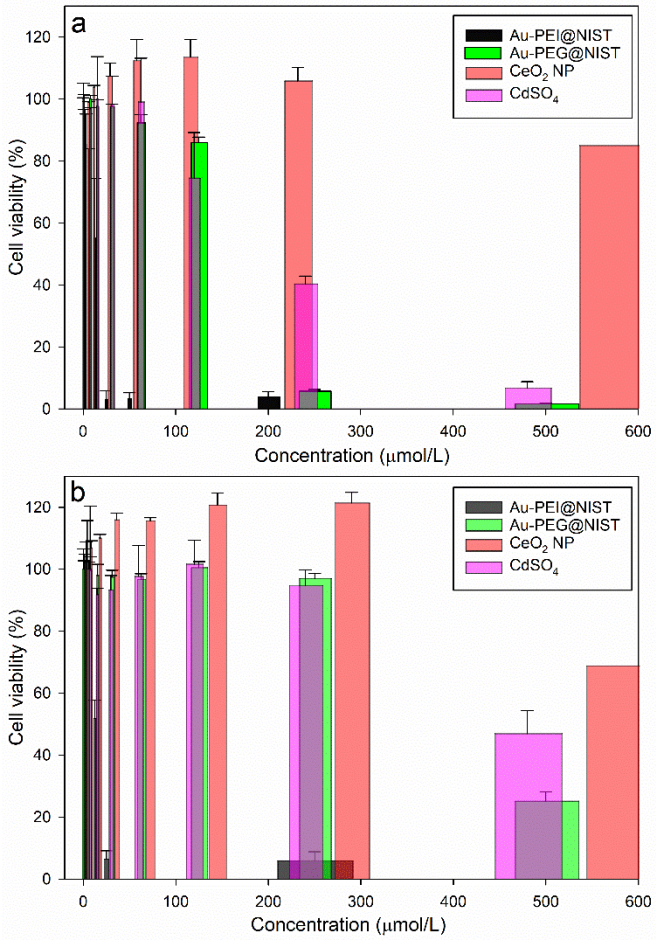

Fig. 3. Representative data of cell toxicity; MTS cell viability assay results for (a) CHO K1, (b) HeLa cell line after 24 h exposure to Au-PEI@NIST nanoparticles (black), CeO_2 NP (red), Au-PEG@NIST (green), and $CdSO_4$ (purple, as a positive control).

IV. SUMMARY

We demonstrate the preparation of 100 mL scale of Au-PEI by optimized condition, using 10 % (mass fraction) of branched PEI (25 kDa) and 2.5 mmol/L of $HAuCl_4$ at a molar ratio ($r^{PEI/Au}$) = 96 and a temperature ramping program of (r.t. to 80) °C for 3 h (after reaching the desired temperature) that resulted in high quality Au-PEI@NIST in terms of narrow size distribution (by DLS, TEM, and AFM), optical properties (SPR by UV-Vis), shape uniformity (by TEM and AFM), and positive surface charge (by ZP). Au-PEI@NIST exhibited long-term colloidal stability at r.t. (for up to 3 years) and exceptional stability in physiologically relevant environment, including biological media, pHs (1.5 ~ 12), and temperatures (20 °C ~ 70 °C). Furthermore, the cytotoxicity of Au-PEI@NIST to CHO K1 (as a normal cell), and HeLa and A549 cells (as cancer cells) were significantly higher than those of the chemical positive control ($CdSO_4$), neutral AuNPs (Au-PEG@NIST), and other type of positively charged nanoparticle (CeO_2). Therefore, our data strongly suggest that Au-PEI@NIST can serve as a robust and efficient nanoscale positive control material for use in nanotoxicology. Also, these results could lead to effective research approaches that could be a clarified starting point for the development of more complex nanomaterials for advanced nanomedicine.

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