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Polyvinyl Alcohol as a Biocompatible Alternative for the Passivation of Gold Nanorods**

*Calum Kinnear, David Burnand, Martin J. D. Clift, Andreas F. M. Kilbinger, Barbara Rothen-Rutishauser, and Alke Petri-Fink**

Abstract: *The functionalization of gold nanorods (GNRs) with polymers is essential for both their colloidal stability and biocompatibility. However, a bilayer of the toxic cationic surfactant cetyl trimethylammonium bromide (CTAB) adsorbed on the nanorods complicates this process. Herein, we report on a strategy for the biocompatible functionalization of GNRs with a hydrophobic polymeric precursor, polyvinyl acetate, which is then transformed into its hydrophilic analogue, polyvinyl alcohol. This polymer was chosen due to its well-established biocompatibility, tunable "stealth" properties, tunable hydrophobicity, and high degree of functionality. The biocompatibility of the functionalized GNRs was tested by exposing them to primary human blood monocyte derived macrophages; the advantages of tunable hydrophobicity were demonstrated with the long-term stable encapsulation of a model hydrophobic drug molecule.*

The great attraction of GNRs stems from their fascinating optical and electrical properties, which are often simply tuned through their size or aspect ratio.[1] With control of these dimensions as a goal, vast improvements have been made over the past 10 years in the reliability of the synthesis leading to monodisperse GNRs and shape yields of close to 100%.[2] As with most nanoparticles (NPs), before use in the desired application, they must first be functionalized with, for example, targeting ligands, inorganic shells, and stabilizing polymers. However, the case of GNRs is notably more complex than that of their spherical analogues, primarily due to the cytotoxic surfactant used and the two distinct surface curvatures at the ends and the sides.[3] Crucially, this surfactant is present as a partially interdigitated bilayer that complicates the functionalization due to the rapid loss of colloidal stability upon destabilization of the bilayer.

Despite the explosion of publications on GNRs (over 7000 from 2002 to 2012 according to the ISI Web of Knowledge), there is typically one polymer used to detoxify the suspension, which is polyethylene glycol (PEG). In a number of other reports either a monomer similar to CTAB was polymerized within the surfactant bilayer,[4] or the CTAB was overcoated with biocompatible polyelectrolytes or amphiphilic polymers such as polyvinylpyrrolidone; however, these reports are few and far between.[5] One reason for the ubiquity of PEG in functionalizing GNRs could be due to its amphiphilic nature, which enables easier penetration through the CTAB bilayer and subsequent grafting to the gold surface with a terminal thiol group. Nevertheless, we have previously shown that the often simple functionalization with thiolated PEG, that is, simply mixing and waiting, is not sufficient especially at low PEG concentrations; ligand exchange occurs in two different stages with the first occurring at the ends of the nanorods before the near-complete surface coverage.[6]

It is essential that alternative polymers are available for passivating the surface of GNRs. This is due to a number of known limitations, four of which we highlight here. Firstly, the PEG coating of "stealth" liposomes has been indicated to cause drug leakage[7] and induce immunogenic responses through complement activation.[8] Secondly, studies have found anti-PEG antibodies in a population of healthy humans; exposure was likely due to the increased use of PEG in pharmaceutical formulations, food products, and cosmetics.[9] These antibodies caused a reduction in the circulation time of PEGylated agents in vivo and accelerated blood clearance upon repeated administration.[10] Thirdly, PEG is known to impart "stealth" properties to NPs, which is potentially undesirable if there is a specific biological target such as the immune system.[11] Finally, there is an ongoing discussion about the significance of tumor targeting via leaky vasculature with "stealth" NPs, as well as the potential side effects of having prolonged exposure to cytotoxic compounds in the blood.[12] In addition to the above limitations, conventional PEG has a low degree of functionality: often only the end group is reactive and therefore further synthetic steps are needed to introduce higher numbers of functional moieties such as dyes or targeting peptides.

It is clear that the ability to test and use alternative polymers is severely hindered by the difficulty of functionalizing GNRs. In order to expand and complement this limited range of biocompatible polymers, we chose to investigate the case of polyvinyl alcohol (PVA). This polymer presents excellent biocompatibility, "stealth" properties dependent on molecular weight, a high degree of hydrophilicity, and tunable hydrophobicity.[13] The combination of these properties along with the FDA approval of PVA in food, pharmaceuticals, and implants such as stents, makes PVA an attractive candidate for GNR functionalization.

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Supporting information for this article is available on the WWW

Taking inspiration from the work of Zubarev et al.[14] and Alkilany et al.,[4] in which a small thiolated analogue of CTAB was used to quantitatively remove the surfactant from the surface of GNRs, we hypothesized that ligand exchange occurs more readily when the surface-adsorbed ligand and the incoming ligand have comparable hydrophobicity. To illustrate this point, we followed a standard one-step method for the PEGylation of CTAB-coated GNRs in water; however, the highly hydrophilic polymer of end-thiolated PVA was used instead of PEG.[6] The exchange was minimal as evidenced by a positive zeta potential (10.4 mV) along with instability at high salt concentrations. Herein we describe the use of a hydrophobic analogue of PVA, end-thiolated polyvinyl acetate (PVAc), to aid the functionalization of GNRs in dimethylformamide (DMF) before hydrolysis of the polymer to transfer the GNRs into an aqueous environment.

The experimental procedure to functionalize and detoxify GNRs is shown in Figure 1 and involves the following steps: 1) GNRs were added dropwise to a stirred solution of PVAc in DMF; the PVAc was synthesized by reversible addition–fragmentation chain-transfer (RAFT) polymerization of vinyl acetate and aminolysis of the RAFT agent to leave a terminal thiol group. 2) The partially functionalized GNRs were transferred to a second solution of PVAc in methanol/ethanol (1:1 v/v) to increase the quantity of adsorbed polymer. 3) Lastly, hydrolysis of the suspension in basic methanol leads to the conversion of PVAc to PVA, resulting in the precipitation of GNRs from suspension due to the limited solubility of PVA in methanol. After the precipitate had been washed multiple times with methanol, the GNRs were readily redispersed by the addition of water and hydration of PVA.

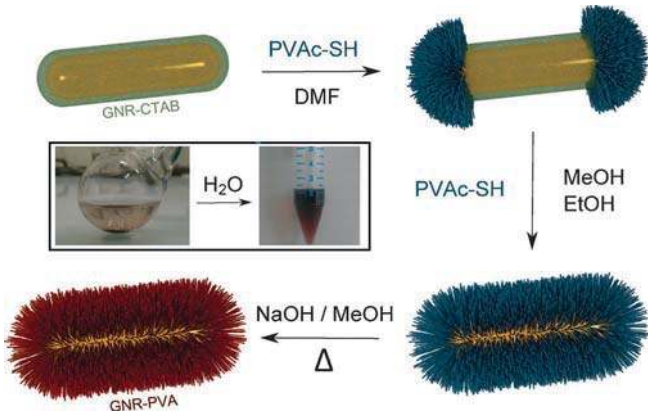
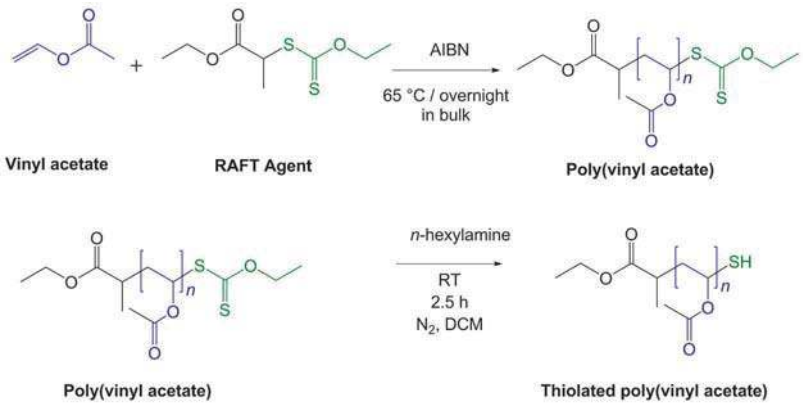


Figure 1. Route to functionalization of GNRs with PVA. A two-step route (reaction with PVAc-SH in DMF, and reaction with PVAc-SH in methanol/ethanol) is used before hydrolysis, which results in the formation of a PVA coating. This renders the GNRs insoluble; precipitation can be easily reversed by the addition of water (inset). The grafting of hydrophobic polymers in DMF at the GNR tips has been shown previously.[16]

GNRs were synthesized in high yield following a previously published route using the cationic surfactant CTAB.[6] The dimensions of the nanorads by transmission electron microscopy (TEM) analysis were 57×12 nm with a 96% shape yield of nanorods relative to spheres. The RAFT-mediated polymerization of vinyl acetate was adapted from a published procedure and gave, after 23 h of bulk polymerization, the desired xanthate end-functionalized polyvinyl acetate (Scheme 1).[15] Gel permeation chromatography in tetrahydrofuran indicated a molecular weight average of 15 kDa and a polydispersity index of 1.3 (see the Supporting Information, SI). The thiolated form was obtained in a one-pot aminolysis of the xanthate moiety under inert atmosphere using *n*-hexylamine. A 100% conversion was found by ^{1}H NMR spectroscopic analysis through the disappearance of the methylene signal of the RAFT agent moiety at 4.7 ppm (SI).



Scheme 1. Synthesis of thiol end-functionalized PVAc by RAFT polymerization. AIBN = azobisisobutyronitrile.

The successful grafting of PVA to the GNRs and the removal of the majority of CTAB was confirmed by a combination of zeta potential measurements, depolarized dynamic light scattering (DDLS), observations of colloidal stability, and the resulting cytotoxicity and pro-inflammatory response of primary macrophages, which we have previously shown to be highly sensitive to residual CTAB.[6] After dispersion of the nanorods in water, the zeta potential was measured as −19.1 mV which is a significant charge reversal compared to the initial value of +35.5 mV before functionalization (SI). The thickness of the PVA layer was estimated by a combination of DDLS and TEM, where the depolarized autocorrelation function was collected at multiple angles using crossed polarizers on a goniometer setup (SI). The resulting decay times were plotted against the wave vector, giving the translational (D_t) and rotational (D_r) diffusion coefficients from the gradient and intercept, respectively, of a linear least-squares fit (Figure 2). A spherocylinder hydrodynamic model was then chosen due to the apparent shape of the gold nanorod from TEM, and the previous success of this model in accurately representing anisotropic nanoparticles.[17]

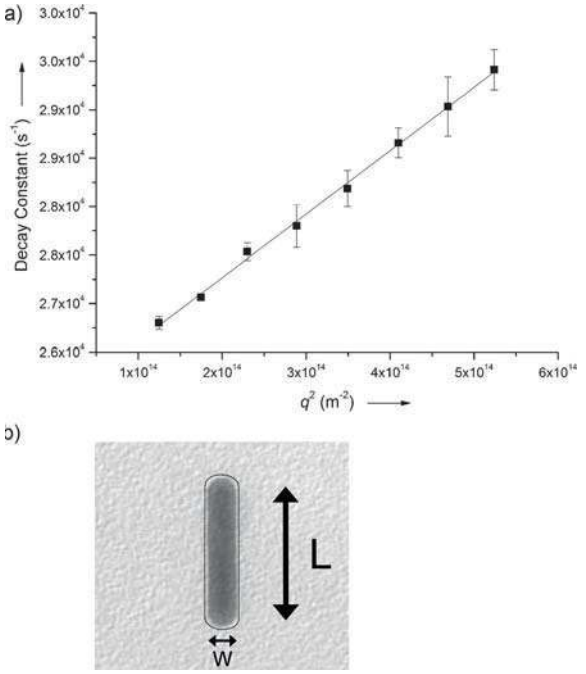


Figure 2. a) Plot of decay constant versus q^2 from DDLS data of PVA-coated GNRs in water at a concentration of 67 μg mL^{-1} at 21 °C. The linear regression (solid line) gives the gradient, equal to D_t, and the intercept, equal to 6 D_r, which are used to calculate the hydrodynamic length and width from a spherocylindrical model (b).

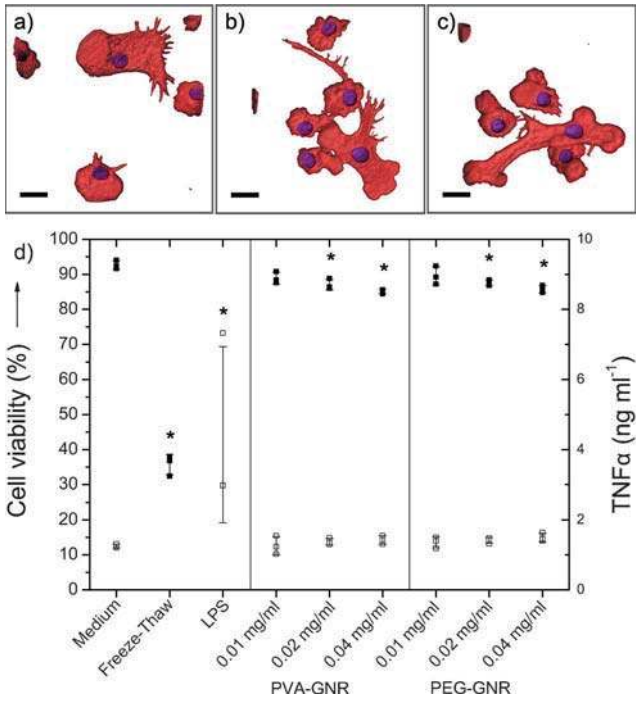


Figure 3. LSM images of human blood monocyte derived macrophages (MDMs) after exposure to a) medium, b) PVA-coated GNRs, and c) PEG-coated GNRs. F-actin is red, nuclei are blue, scale bar represents 25 μm. d) MDM viability determined by the Trypan blue assay (■) and release of proinflammatory cytokine tumor necrosis factor (TNF-α) determined by ELISA after 24 h suspension exposure to PVA- or PEG-coated GNRs (□). Cell samples treated with a freeze–thaw cycle, −80 °C for 30 min, and lipopolysaccharide (LPS) 0.1 mg mL^{-1}, served as positive controls. A two-way Tukey post-hoc ANOVA was performed and significance is indicated by: $*p < 0.05$ versus the negative control.

This model was used to estimate the hydrodynamic length and width of the GNRs (SI). The measured dimensions from TEM were then subtracted from the length and width obtained by DDLS, giving an estimate for the thickness of the PVA coating: approximately 13 nm at the tips and 18 nm at the sides. This is greater than the known thickness of a CTAB bilayer, 3.4 nm, indicating the presence of a polymeric coating.[18]

We qualitatively assessed the hydrophobicity of GNRs after the first functionalization step with PVAc in DMF; if the coating was successful, then the GNRs should be water insoluble. As predicted, at low concentrations of water in DMF (10 % v/v), the GNRs instantaneously aggregated (Figure S8, SI). Notably, colloidal stability is lost if CTAB-GNRs in aqueous suspension are added directly to a solution of PVAc in ethanol. Additionally, without the second functionalization step in ethanol, after DMF, the PVA-GNRs are more aggregated (Figure S10, SI). Given these results, along with the solubility of the PVAc-GNRs in ethanol and their resulting water solubility after hydrolysis, it is reasonable to conclude that the GNRs were functionalized with PVA and that a large quantity of the CTAB was removed.

The ultimate goal of the surface functionalization of GNRs is their detoxification for therapy, sensing, or theranostic applications.[19] Therefore, we tested their biocompatibility by exposing PVA-GNRs at three different concentrations over 24 h to human blood monocyte derived macrophages (MDMs) alongside a negative control of PEGylated GNRs, which we have previously shown to be non-cytotoxic and elicit limited pro-inflammatory cytokine release in vitro.[6] The results of these experiments are shown in Figure 3, where a significant but small decrease in cellular viability relative to the negative control was found for the two highest concentrations (0.02 and 0.04 mg mL^{-1}) of the two functionalized GNRs, as measured by trypan blue exclusion. These findings were qualitatively complemented by laser scanning confocal microscopy (LSM) where no changes in morphology of the macrophages were observed for either of the two GNR samples even at the highest exposed dose. Additionally, neither sample resulted in an increase in the production of the pro-inflammatory cytokine TNF-α relative to the negative control.

The hydrophobicity of PVA can be tuned by quenching the hydrolysis reaction before completion.[20] We exploited this property by trapping a hydrophobic dye in PVA-GNRs and chemically triggering its release, with a view to potential applications in hydrophobic drug delivery. Specifically, we performed the hydrolysis reaction in the presence of the hydrophobic fluorophore Nile Red and after the flocculate had been washed once with methanol, water was added driving the Nile Red into the remaining hydrophobic pockets in the polymeric layer. Free dye was removed by multiple rounds of centrifugation. Following this, the GNRs were mixed by vortex with an equal volume of chloroform. After four weeks, there was no detectable dye in the organic phase;

however, upon addition of mercaptoethanol, which is known to form a monolayer on the surface of gold NPs and thereby displace the polymer, a significant release was immediately observed (Figure 4).[21] Quantification of the released dye by fluorescence spectroscopy indicated that at least 3000 molecules were trapped within each polymeric shell (SI). Interestingly, the fluorescence was completely quenched when the

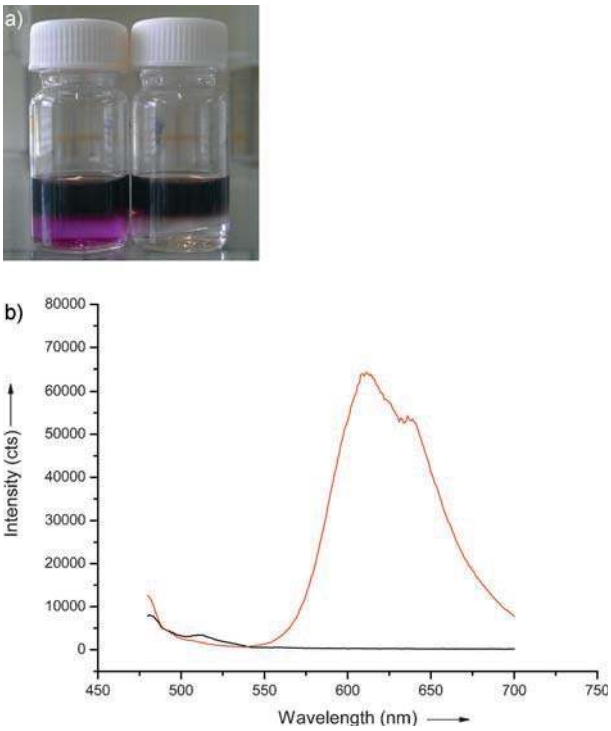


Figure 4. a) Release of a trapped hydrophobic dye from an amphiphilic polymeric shell surrounding a GNR in water to the organic phase (chloroform). The trigger used here was mercaptoethanol. b) Emission spectra of PVA-coated GNRs with trapped dye, Nile Red, before and after addition of mercaptoethanol. Quantification by fluorescence spectroscopy indicated at least 3000 dye molecules were attached to each GNR.

dye was attached to the GNR, in an aqueous dispersion; the peak around 520 nm in the emission spectra originates from enhanced Rayleigh scattering near the plasmon energy (Figure 4b). In a further control experiment the GNRs were incubated in fetal bovine serum to determine whether biological conditions are able to displace the PVA or remove the Nile Red. We observed a small quantity of dye released to the organic phase; however, quantification was complicated by the stabilizing effect of the proteins leading to the formation of an emulsion. Nevertheless, this proof of concept highlights one of the advantages of using partially hydrolyzed PVA for the application of poorly soluble pharmaceuticals—a major challenge for the application of most class II and IV drugs (Biopharmaceutics Classification System).[22].

In summary, the procedure reported herein enables the functionalization of CTAB-coated GNRs with a highly hydrophilic polymer through first attaching a hydrophobic analogue in a common solvent. The subsequent hydrolysis reaction generates a highly hydrophilic PVA coating, which has both tunable hydrophobicity and a high degree of functionality. The encapsulation of a model hydrophobic compound was used to illustrate the advantage of the remaining hydrophobic pockets on the surface of the GNRs. Hypothetically, the presence of one hydroxy group in every monomer for PVA far outweighs the single end-functional group of PEG in terms of degrees of functionality. Additionally, the "stealth" properties of PVA are known to depend on its molecular weight, with larger polymers exhibiting in vivo circulation times such that circulation times can be tuned. All of these points lead us to highlight the complementarity of PVA and PEG in functionalizing NPs. The route to PVA-GNRs could in theory be applied to similar hydrophobic–hydrophilic analogues, and complements the limited library of polymers that are currently used to successfully detoxify and functionalize GNRs.

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