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Rapid determination of plasmonic nanoparticle agglomeration status in blood

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ABSTRACT

Plasmonic nanomaterials as drug delivery or bio-imaging agents are typically introduced to biological systems through intravenous administration. However, the potential for agglomeration of nanoparticles in biological systems could dramatically affect their pharmacokinetic profile and toxic potential. Development of rapid screening methods to evaluate agglomeration is urgently needed to monitor the physical nature of nanoparticles as they are introduced into blood. Here, we establish novel methods using darkfield microscopy with hyperspectral detection (hsDFM), single particle inductively-coupled plasma mass spectrometry (spICP-MS), and confocal Raman microscopy (cRM) to discriminate gold nanoparticles (AuNPs) and their agglomerates in blood. Rich information about nanoparticle agglomeration *in situ* is provided by hsDFM monitoring of the plasmon resonance of primary nanoparticles and their agglomerates in whole blood; cRM is an effective complement to hsDFM to detect AuNP agglomerates in minimally manipulated samples. The AuNPs and the particle agglomerates were further distinguished in blood for the first time by quantification of particle mass using spICP-MS with excellent sensitivity and specificity. Furthermore, the agglomeration status of synthesized and commercial NPs incubated in blood was successfully assessed using the developed methods. Together, these complementary methods enable rapid determination of the agglomeration status of plasmonic nanomaterials in biological systems, specifically blood.

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1. Introduction

Nanomaterial-containing commercial products are under intense development by the pharmaceutical industry for imaging, diagnosis, prevention, and treatment of disease [1]. The safety of these new, nanomaterial-containing products remains a concern to scientists and the public [2]. The toxicity of nanomaterials depends strongly on their physiochemical properties (*e.g.* size, size distribution, shape, surface charge, crystal structure, hydrophobicity, surface reactivity, solubility, aggregation and purity) and material composition itself [3–5]. The United States Food and Drug Administration (FDA) has developed a nanotechnology regulatory science program to enhance research in nanomaterial characterization, *in vitro* and *in vivo* modeling, and product-focused disposition and toxicity [6]. One priority is the development of the analytical tools to detect and characterize nanomaterials in commercial products, food matrices, and biological systems. The challenge facing biomedical research is the poor understanding of the agglomeration status and biological fate of nanomaterials once they are introduced into the blood stream.

Gold nanomaterials (GNMs) are particularly appealing candidates as new diagnostic and therapeutic agents because of their relative bioinertness, tightly controllable morphology, facile surface functionalization, and unique optical properties [7–10]. Some GNM-based medicines are in clinical trials. As an example, Aurimune® is a nanomedicine with tumor necrosis factor (rhTNFα)

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covalently conjugated to PEGylated gold nanoparticles that has been approved for phase II clinical trials for cancer therapy [11]. AuroLase®, which is also in clinical trials, utilizes gold nanoshells and laser technology as a new photothermal treatment modality for refractory head and neck cancer [12]. In practice, GNM-based medicine is generally administered intravenously, *i.e.* directly into the blood stream. Blood is a complex fluid with significant concentrations of proteins, salts, and blood cells. Nanoparticles interact with blood proteins; adsorption onto the materials' surface results in the formation of a protein corona [13,14]. The change in surface properties introduced by blood ionic strength can induce nanoparticle agglomeration [15], and thus significantly alter the cellular interactions, biodistribution [16], and toxicity profile of the particles [17]. Additionally, nanomaterials retard cell motility [18], and nanomaterial induced endothelial leakage (NanoEL), wherein nanomaterials bypass the cell membrane by disrupting through adherens junction, has also shown a strong correlation to the hydrodynamic size of the particle [19]. Due to their potential for agglomeration, *in situ* monitoring of nanoparticles in blood is crucial to fully understand *in vivo* effects of GNM-materials targeted for human theranostics.

There are many well-developed methods to characterize GNMs; however, characterizing GNMs in blood is complicated by the complex environment, *e.g.* plasma proteins and various blood cells. Electron microscopy (EM) is considered the most accurate method to measure the size of nanoparticles and the most widely used method of assessing nanoparticle morphology. However, EM requires considerable sample manipulation, which introduces artifacts; it provides only a static image of the GNMs, typically in thin, dry sections of tissues or matrices (*e.g.* 50–100 nm), and cannot readily distinguish agglomerates from primary particles located in close proximity [20–23]. Recently, cellular uptake of 30 nm, spherical AuNPs has been visualized using scanning transmission electron microscopy (STEM) imaging of liquids, yet at this point the technique suffers from low resolution [24]. Chromatographic techniques, such as size-exclusion chromatography, ultracentrifugation, and electrophoresis, have been shown to separate primary and agglomerated nanoparticles based on shape, size, or charge [25]. One considerable disadvantage is that these techniques often require extensive sample preparation, which may affect particle agglomeration status. Dynamic light scattering (DLS) has been used to estimate particle concentration in blood [26]; however, DLS is limited due to (1) its requirement that the blood cells to be lysed prior to measurement, which could affect the agglomeration status of nanomaterials; (2) the presence of proteins and cells in blood interferes with nanoparticle signal; and (3) larger particles which are overrepresented in polydisperse samples [27]. UV–Vis and Raman spectroscopy can readily distinguish primary and agglomerated plasmonic nanoparticles [28,29], but the signal is compromised by the extremely high optical density and opacity of blood. Accordingly, we are unable to find a simple and robust methodology for *in situ* characterization of nanoparticle agglomeration in blood and other complex biological environments.

In this work, complementary and rapid *in situ* methods have been developed to monitor the agglomeration status of plasmonic nanoparticles in *ex vivo* blood. These methods include darkfield microscopy with hyperspectral imaging (hsDFM), confocal Raman microscopy (cRM), and single particle inductively-coupled plasma mass spectrometry (spICP-MS). The first two methods are based on the optical properties of GNMs, that is, the localized surface plasmon resonance (LSPR) [30]. The LSPR is extremely sensitive to particle morphology with agglomeration of GNMs resulting in a shift of the LSPR to lower energy [31]. The hsDFM can monitor this shift of scattered photons from GNMs, thereby distinguishing light scattered by the cellular environment from that scattered by nanoparticles [32–36], and this instrument has been used to distinguish macrophage maturity based on silver nanoparticle (AgNPs) uptake [37]. Scattering also gives rise to enhancement of the fluorescence and Raman scattering properties of molecules close to the GNMs [38]. The areas of high curvature or between adjacent AuNPs at agglomeration sites create localized "hot spots" that enhance Raman signals, similar to a roughened metal surface [38], with enhancement factors that can be 10^8 or greater [39]. Surface enhanced Raman spectroscopy (SERS) has been used to identify circulating tumor cells in blood [40] and detect tumor cells *in vivo* [41], as well as a wide-range of other chemical detection and sensing applications [42,43]. We have applied SERS *via* cRM to directly monitor nanoparticle agglomeration as a label-free detection method in this study.

In addition to the optical techniques, spICP-MS provides a high-resolution technique to detect individual particle events for characterization of a variety of engineered nanomaterials [44]. Unlike traditional ICP-MS, which provides bulk elemental analysis of homogenized metallic species, spICP-MS provides a measure of individual particles in suspension. At sufficiently low particle concentrations, particles are introduced individually for atomization and ionization in the plasma, creating a packet of ions that are detected as a discrete signal. This quantifiable spike in intensity due to single particle events enables individual nanoparticles to be distinguished from the background ionic current [45]. The signal intensity is proportional to the number of atoms in an individual event, so larger particles produce higher signal intensity. Similarly, particle agglomeration can be detected by monitoring the increase in signal intensity [46]. By collecting a large number of data points, the particle size and distribution can be determined [44,46–48].

In this report, we have validated and applied the spICP-MS technique to analyze the agglomeration status of nanoparticles in blood as a complement to the optical methods. AuNPs and agglomerates were synthesized and characterized using well-developed methods like UV–Vis, Raman spectroscopy, TEM, DLS, nanoparticle tracking analysis (NTA), and discrete dipole approximation (DDA) calculations. Detection of particle agglomeration was validated in a variety of simple biological media. The promising methods were then used to detect agglomerates in blood, with hsDFM and spICP-MS as the primary methodologies. Finally, citrate-capped NPs were incubated in blood, and their agglomeration was monitored using the developed methods. Together, hsDFM, cRM, and spICP-MS provide a rapid and robust means to analyze nanoparticle agglomeration in biological systems with minimal sample preparation. These diverse, label-free methods can distinguish primary particles from agglomerates in blood. Identification of the interactions between nanoparticles and components in biological systems is, in turn, critically important for rational design and implementation of nanomedicine.

2. Materials and methods

2.1. Chemicals

Tetrachloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$) and trisodium citrate heptahydrate (Na_3-Cit; $Na_3C_6H_5O_7 \cdot 7H_2O$) were acquired from Alfa Aesar (Ward Hill, MA). Phosphate buffered saline (PBS), fetal bovine serum (FBS), and Eagle medium were acquired from Corning Cellgro (Manassas, VA). The reference AuNPs with nominal diameters of 10 nm (RM8010), 30 nm (RM8012), and 60 nm (RM8013), were purchased from the National Institute of Standards and Technology (NIST). Citrate-capped 75-nm AgNPs were obtained from NanoComposix (San Diego, CA). Sodium chloride (NaCl) and bovine serum albumin (BSA) were obtained from Sigma Aldrich (St. Louis, MO). All chemicals were used as received.

2.2. Synthesis of AuNPs and agglomerates

Citrate-capped AuNPs (AuNP-cit) were synthesized *via* the Turkevich method as previously described [49]. Briefly, 10.6 mg $HAuCl_4$ was dissolved in 99 mL 18 MΩ H_2O and heated to boiling. To this solution, 0.9 mL of 10 mg/mL Na_3Cit was quickly injected. After 20 min, a deep wine-red color was observed, indicating the formation