N-Heterocyclic Carbene Ligand Stability on Gold Nanoparticles in Biological Media

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ABSTRACT: The ability to functionalize gold nanoparticle surfaces with target ligands is integral to developing effective nanosystems for biomedical applications, ranging from point-of-care diagnostic devices to site-specific cancer therapies. By forming strong covalent bonds with gold, thiol functionalities can easily link molecules of interest to nanoparticle surfaces. Unfortunately, thiols are inherently prone to oxidative degradation in many biologically relevant conditions, which limits their broader use as surface ligands in commercial assays. Recently, N-heterocyclic carbene (NHC) ligands emerged as a promising alternative to thiols since initial reports demonstrated their remarkable stability against ligand displacement and stronger metal–ligand bonds. This work explores the long-term stability of NHC-functionalized gold nanoparticles suspended in five common biological media: phosphate-buffered saline, tris-glycine potassium buffer, tris-glycine potassium magnesium buffer, cell culture media, and human serum. The NHCs on gold nanoparticles were probed with surface-enhanced Raman spectroscopy (SERS) and X-ray photoelectron spectroscopy (XPS). SERS is useful for monitoring the degradation of surface-bound species because the resulting vibrational modes are highly sensitive to changes in ligand adsorption. Our measurements indicate that imidazole-based NHCs remain stable on gold nanoparticles over the 21 days of examination in all tested environments, with no observed change in the molecule’s SERS signature, XPS response, or UV–vis plasmon band.

INTRODUCTION

Gold nanoparticles (AuNPs) are commonly employed for bionanotechnology applications due to their facile synthesis, high surface areas, tunable optical properties, and low cytotoxicity.1 Along with these intrinsic advantages of AuNPs, most nanoparticle-based systems require an additional component: strategically chosen surface ligands. For example, nanoparticle surfaces functionalized with biological recognition elements, such as DNA, peptides, antibodies, or aptamers,2 are often crucial for disease diagnostics and targeted drug therapies.3

Traditionally, AuNP systems almost exclusively utilize thiol groups as surface functionalization agents to tether requisite biomolecules because thiols form a covalent bond with the gold surface and self-assemble into highly ordered monolayers.4 Thiol linkages underpin DNA-conjugated nanostructures,5 anchored photosensitizers for photodynamic therapy to treat antibiotic-resistant bacterial infections,6 and aptamer-mediated drug delivery strategies.7 Nevertheless, while thiol functionalities have been integral for advancing bionanotechnology applications, their instability in biologically relevant conditions has been well documented,8 motivating the search for more robust alternative ligands.9

In the early 2010s, several researchers explored the potential application of N-heterocyclic carbenes (NHCs) as functionalization agents for gold surfaces,10 and in 2014, Crudden and co-workers reported that NHCs form compact, self-assembled monolayers on Au(111) that resist ligand displacement.11 NHCs have since become attractive ligands for functionalizing nanoparticle surfaces with applications ranging from catalysis12 to biomedicine.13 Due to their strong σ-donation, NHCs readily bind to transition metals resulting in highly robust complexes,14 which have been used as precursors for appending NHCs to nanoparticles.15–17 Still, for this new class of surface ligands to achieve widespread use in biotechnology, their stability must first be tested in biologically relevant conditions.

For nanoparticle-based systems, stability assessments can be divided into two questions (Figure 1): first, do the functionalized particles aggregate in solution? Second, does the ligand dissociate from the surface over time? To date, aqueous NHC gold nanoparticle stability investigations have only addressed the first question.16,18,19 For example, gold nanoparticle systems with NHC ligands have exhibited colloidal stability in aqueous environments (pH 3–14), high salt solutions, extreme temperatures, phosphate-buffered saline (PBS), and cell culture media.16,18,19 These studies primarily employed...
UV−vis spectroscopy, supplemented by electron microscopy (EM) imaging and dynamic light scattering (DLS) techniques, to track the plasmon band as nanoparticles were exposed to different environments (Figure 1, Question 1). The plasmon band position is dependent on the size and shape of nanoparticles, which makes it a convenient method for studying aggregation.

To probe the impact of the environment on ligand dissociation (Figure 1, Question 2), previous work has focused on assessing the integrity of the ligand-functionalized surface by employing X-ray photoelectron spectroscopy (XPS) and surface-enhanced Raman spectroscopy (SERS). XPS analysis elucidates the elemental composition and chemical bonding states present at a material’s surface. SERS provides real-time measurements of the chemical environment near the nanoparticle. In addition, SERS is highly sensitive to changes in ligand adsorption and orientation on the substrate surface, which can be observed in the resulting vibrational spectra. NMR characterization was explored by Crespo and co-workers to analyze the surface state of ultrasmall (<2 nm in diameter) nanoparticles functionalized with NHCs. So far, NHC ligands appended to solid gold substrates have been shown to be robust in extreme pHs, temperatures exceeding the physiological range, and oxidizing conditions. However, there have been no reports to date investigating ligand dissociation of NHCs in solution-phase nanoparticle systems exposed to biologically relevant conditions. Further investigations are needed to demonstrate the practical utility of NHC-AuNP systems.

Herein, we investigate the long-term stability of NHC ligands adsorbed on gold nanoparticles when subjected to biological media that are routinely used for developing medical diagnostics and therapeutics. The five media tested, by increasing molecular complexity, are phosphate-buffered saline (PBS), tris-glycine potassium (TGK) buffer, tris-glycine potassium magnesium (TGKM) buffer, cell culture media, and human serum. The robustness of the ligand−gold bond is monitored primarily with SERS, supplemented by XPS characterization where applicable. In this work, we observe no dissociation of the imidazole-based NHC ligand on the AuNP surface in all tested conditions over a 21 day period, including, remarkably, full-strength human serum.

■ RESULTS AND DISCUSSION

The citrate-capped AuNPs exhibit a sharp plasmon band at 521 nm measured with UV−vis spectroscopy (Figure 2a). The hydrodynamic diameter output by dynamic light scattering (DLS) depends on both the core nanoparticle size as well as any surface coating that affects the movement of particles in fluid. Therefore, it is expected that calculated diameters from DLS will be slightly larger than those derived from scanning electron microscopy (SEM) images for the same functionalized AuNPs, which we consistently observed. Zeta-potentials (ZPs) are deduced from measuring velocities of charged particles under an applied electric field and can be an indication of colloid stability in suspended media. In general, increasing ZP magnitude is associated with higher stabilization in solution. With an absolute value of ∼20 mV, our AuNPs are considered moderately stable. SEM and transmission electron microscopy (TEM) imaging (Figure 2b) demonstrate that the particles are monodisperse, spherical, and approximately 19 nm in diameter, which is consistent with the UV−vis, DLS, and ZP analyses. The size distribution (Figure 2c) was determined by

![Figure 1. Scheme depicting two questions for assessing the stability of functionalized nanoparticle systems. Previous work focused on the extent of aggregation (1), while this work focuses on the nanoparticle−ligand interaction in a variety of biological media (2).](image1)

![Figure 2. Characterization of citrate-capped AuNPs prior to NHC functionalization. (a) UV−vis spectrum of prepared nanoparticles suspended in water accompanied by DLS and ζ-potential measurements. (b) Representative SEM and TEM (inset) images of spherical AuNPs. (c) Histogram depicting nanoparticle size distribution of a representative colloid batch, based on the analysis of 206 imaged particles. The reported errors are standard deviations based on three replicate measurements.](image2)
analyzing five independent SEM image segments with the Image Processing Toolbox in MatLab (Version 2020b, MathWorks).

Two NHC-Au precursor complexes, [bis(1,3-bis(isopropyl)-imidazol-2-ylidene)gold(I)] chloride and [bis(1,3-diisopropyl-benzimidazolin-2-ylidene)gold(I)](hexafluorophosphate), were synthesized following previously established protocols.24,25 The resulting 1H and 13C NMR spectra matched previous reports24,25 and display high purity (Figures S1−S6). AuNPs can then be easily functionalized following a benchtop method by Camden, Jenkins, and co-workers,15 whereby exposing citrate-capped AuNPs to a solution of bis-NHC-Au complex in dichloromethane quickly forms chemisorbed NHC-AuNPs (Figure 3).

Figure 4 presents characterization data for the nanoparticles after functionalization. The UV−vis spectrum is relatively unchanged with a single, sharp peak at 522 nm, while DLS measurements indicate that the nanoparticle size is unaffected by functionalization, consistent with previously reported TEM and SEM images.15 Taken together, these results demonstrate that NHC-AuNPs are relatively stable with no observed aggregation. However, the decreased ZP magnitude indicates that the functionalized particles (Figure 4) may be less stable due to a dihedral effect, as shown in our previous study.29 Interestingly, our spectra of imidazole on AuNPs show these bands as reference between the previously reported spectra and the signatures reported here: rather than the 1325 cm−1 band being much stronger than the 1291 cm−1 band, our spectra of imidazole on AuNPs show these bands as relatively equal in intensity (Figure 5a). This change may be due to a difference in the environment of the isopropyl side groups.29 Specifically, our spectra exhibit these bands at 1291, 1325, and 1401 cm−1, which corresponds well with previously reported SERS spectra.27,28 The resulting SERS spectra are also in agreement with previously reported benzimidazole NHC vibrational signatures.27 Over the course of 21 days, we observed no changes to the UV−vis spectra and the signatures reported here: rather than the 1325 cm−1 band being much stronger than the 1291 cm−1 band, our spectra of imidazole on AuNPs show these bands as relatively equal in intensity (Figure 5a). This change may be due to a difference in the binding of ligands to the substrate surface between roughened solid Au and AuNPs, which could affect the average configuration of isopropyl side groups.29

At 7 day intervals, the NHC-AuNP solutions in PBS were investigated using SERS (Figure 5). The vibrational modes of imidazole NHC on gold surfaces have been previously characterized by a dual experimental and theoretical approach.27 The strong band at 1555 cm−1 is associated primarily with imidazole ring hydrogens (Figure 5a). The set of bands between 1250 and 1450 cm−1 are due to a complex coupling of the isopropyl side group hydrogens to heterocycle hydrogens, where the resulting normal modes are affected by the orientation of side groups with respect to the surface (i.e., pointing upwards or downwards).27,28 Specifically, our spectra exhibit these bands at 1291, 1325, and 1401 cm−1, which corresponds well with previously reported SERS spectra.27,28 There is one consistent difference between the previously reported spectra and the signatures reported here: rather than the 1325 cm−1 band being much stronger than the 1291 cm−1 band, our spectra of imidazole on AuNPs show these bands as relatively equal in intensity (Figure 5a). This change may be due to a difference in the environment of the isopropyl side groups.29 Over the course of 21 days, we observed no changes to the SERS signature of imidazole NHC on AuNPs when suspended in PBS (Figure 5a). This result indicates that the ligand binding is unchanged, as SERS is highly sensitive to changes in the adsorption of molecular species. As an additional test, we functionalized AuNPs with a different NHC ligand, benzimidazole, and resuspended those particles in PBS (Figure 5b). The resulting SERS spectra are also in agreement with previously reported benzimidazole NHC vibrational signatures.15,27 Similarly, no changes in the benzimidazole NHC ligand SERS spectra were observed over 21 days, indicating minimal degradation of the surface-bound species (Figure 5b).
These stability experiments were repeated in four additional media: TGK buffer, TGKM buffer, cell culture media, and full-strength human serum (Figure 6). The remaining studies used the imidazole NHC ligand for nanoparticle functionalization. In all cases, the imidazole NHC vibrational signature is preserved, demonstrating that nanoparticle suspension in these biological media does not result in significant changes to surface-bound ligands over the observation period (Figure 6). We observe no signal loss in PBS (Figure S7) and human serum (Figure S8).

As an additional test to probe the ligand stability, we characterized the imidazole NHC-AuNP system with X-ray photoelectron spectroscopy (XPS). XPS is a standard approach for investigating the chemical states of ligands on metal surfaces. In particular, the appearance of a N 1s peak between 399 and 401 eVs is used to indicate the presence of chemisorbed NHCs. The characteristic N 1s XPS peak for imidazole NHC-AuNPs (Figure 7a) remains unchanged during the 21 day observation period for NPs suspended in both water and PBS buffer (Figures S9 and 7b, respectively), providing further evidence that the NHC ligand is unchanged on the nanoparticle surface in this media. No nitrogen signal was observed when citrate-capped AuNPs were exposed to PBS buffer (Figure 7a). The Au4f XPS spectra were similar and consistent with previous reports (Figure S10). Unfortunately, meaningful data could not be obtained for TGK buffer, TGKM buffer, cell culture media, and human serum due to signal contamination from nitrogen in the biological media. Taken together, the SERS and XPS data indicate that NHC ligands remain chemisorbed to AuNPs for at least 3 weeks in a variety of commonly used biological media.

While nanoparticle stability in suspension is not the focus of this work, we did perform UV−vis experiments over 21 days of imidazole NHC-Au-NPs in PBS buffer, TGK buffer, cell culture media, and human serum (Figure S11). Unsurprisingly, there were signs of aggregation in the concentrated salt buffers. However, NHC-AuNPs were stable over time in the cell culture media and human serum (Figure S11), displaying no peak shifting or broadening of the dominant plasmon band. We attribute this continued suspension in solution to the formation of a protein corona, as has been previously reported.32

**CONCLUSIONS**

We report the first systematic examination of NHC ligand stability on gold nanoparticles in a number of media relevant to biomedical applications. SERS and XPS data suggest that
imidazole NHC ligands remain intact on gold nanoparticles for at least 21 days under these conditions, including full-strength human serum. We believe that this work will further motivate research efforts exploring the implementation of these robust ligands in biological assays and nano-based therapeutic systems.

**EXPERIMENTAL SECTION**

**Preparation of Biological Media.** Phosphate-buffered saline (PBS, pH 7.2, Gibco) and human serum (male, type AB, sterile, Fisher BioReagents) were purchased from Fisher Scientific. Tris-glycine potassium (TGK) buffer (25 mM tris-HCl, 192 mM glycine, and 5 mM KH2PO4, pH 8.3) was made from a 10× solution of tris-glycine (VWR) and solid KH2PO4 (VWR). Tris-glycine potassium magnesium (TGKM) buffer was prepared from TGK buffer with the addition of magnesium chloride to a final Mg2+ concentration of 5 mM (VWR). Cell culture media consisted of RPMI 1640 (VWR) and L-glutamine (VWR). Reagents were used as received without further purification.

**Synthesis of NHC Gold Complexes.** [Bis(1,3-bis(isopropyl)imidazol-2-ylidene)gold(I)]chloride was synthesized from chloro(dimethyl sulfide)gold, 1,3-diisopropylimidazolium iodide, and KN(SiMe3)2 by the method of Baker.24 [Bis(1,3-diisopropylbenzimidazolin-2-ylidene)gold(I)]- (hexafluorophosphate) was synthesized from chloro(1,3-diisopropylbenzimidazolin-2-ylidene)gold(I), 1,3-diisopropylbenzimidazolium hexafluorophosphate, and K2CO3 by the method of Huynh.25 1H and 13C NMR spectra matched previously published reports. The mono-NHC complexes can be differentiated from bis-NHC complexes by comparison of the carbene resonance in 13C NMR.24,25,33

**Preparation of NHC-Functionalized AuNPs Suspended in Biological Media.** Citrate-capped gold nanoparticles (AuNPs) were prepared by following the Lee and Meisel protocol.15 In short, 274 mg of HAuCl4·3H2O was added to 500 mL of pure water (18.2 MΩ-cm, Barnstead Nanopure System, Thermo Fisher Scientific) in an Erlenmeyer flask. The stirring solution was brought to a vigorous boil. Then, 10 mL of freshly prepared 1% sodium citrate solution (561 mg in 50 mL of H2O) was added. Upon observing a distinctive color change from colorless to wine red, the reaction conditions were maintained for an additional 60 min. The flask was removed from the heat and allowed to cool. Last, water was added to reach the 1000 mL fill line. The synthesized particles were approximately 19 nm in diameter with a characteristic plasmon band at 521 nm, measured by SEM and UV–vis, respectively. The colloids were stored at room temperature in a dark cabinet prior to use.

NHC-functionalized AuNPs were synthesized according to a method previously described by Camden, Jenkins, and co-workers,15 whereby NHC-Au complexes undergo rapid ligand exchange with citrate molecules capping the nanoparticle surface, resulting in chemisorbed NHC-AuNPs. To a vial containing 5 mL prepared citrate-capped AuNP solution, a 5 μL aliquot of NHC-Au complex stock (1 mM in dichloromethane) was added. Upon mixing for 20 min in ambient conditions, NHC-functionalized aqueous AuNPs were formed.

Nano-particle solutions were centrifuged for 30 min followed by removal of the aqueous supernatant, isolating NHC-AuNPs. To maintain the same concentration of particles, 5 mL of the desired biological media was added to the centrifuge tube. The mixture was sonicated to resuspend the nanoparticles in the media. Five different conditions were tested: PBS buffer, TGK buffer, TGKM buffer, cell culture media, and human serum. The prepared solutions were stored in glass vials at 6 °C for 7, 14, or 21 days. To facilitate SERS analysis, on the specified day, 1 mL of 1 M sodium bromide (NaBr) was added to the sample vial and allowed to mix for 10 min, aggregating the nanoparticles. The resulting aggregates were immediately analyzed via SERS. For XPS analysis, samples were freeze-dried for 24 h, yielding a powder. The solidified nanoparticles were mounted on a 60 mm platen with a double-sided conductive copper tape.

**Instrumentation.** Dynamic light scattering (DLS) and ζ-potential measurements were taken with a NanoBrook Omni Instrument (Brookhaven Instruments Corporation) equipped with a 35 mW diode laser (λ = 640 nm). DLS measurements were performed with a 90° scattering angle. The phase analysis light scattering (PALS) technique was employed for the ζ-potential analysis. The reported means and standard deviations are based on three replicate measurements, each with 100 s acquisition time.

SERS measurements were obtained with a custom-built Raman instrument. A HeNe laser (λ = 633 nm, Thor Labs) was focused onto the sample with an inverted microscope objective (Nikon, 20×, NA = 0.5). At the objective, there was approximately 800 μW of power. Each scan was acquired for 2 min. The resulting scattered light was accumulated in a

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Figure 7. (a) XPS controls. N 1s spectra of citrate-capped AuNPs in PBS buffer (blue trace) and imidazole NHC-AuNPs in water (green trace) along with the peak fit (black trace). (b) XPS spectra of imidazole NHC-AuNPs suspended in PBS buffer at day 0 (green traces) and day 21 (red traces). The overall fits for N 1s and C 1s are all solid black lines.
backscattering geometry, passed through a Rayleigh rejection filter (Semrock), dispersed with a spectrometer (Acton SP2300, Princeton Instruments, 1200 g mm \(^{-1}\)), and detected with a back-illuminated, deep-depletion CCD (PIXIS, Spec-10, Princeton Instruments). The scans were analyzed with Winspec 32 software (Princeton Instruments). Each spectrum is an average of three scans taken at different colloid aggregate positions, which were plotted in IGOR Pro (Wavemetrics).

A VersaProbe II Instrument (Physical Electronics), equipped with a monochromatic Al Kα X-ray source (photon energy = 1486.6 eV), was used for the XPS analyses. High-resolution spectra were recorded under ultrahigh vacuum with a 23.50 eV pass energy. The data was analyzed using MultiPak software (Physical Electronics), where the scans were calibrated to the C 1s binding energy at 284.8 eV and background subtracted following the Shirley algorithm.\(^\text{35}\)

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c06168.

NMR, SERS, XPS, and UV–vis spectra, Figures S1–S11 (PDF)

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**Author Contributions**

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This was supported by the National Science Foundation under grant numbers CHE-2108330 (L.M.S., M.D.F., and J.P.C.) and CHE-2108328 (S.L.S., R.K.B., and D.M.J.). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. In addition, this work was supported by the Institute for Precision Health (formerly, Advanced Diagnostics and Therapeutics) at the University of Notre Dame (ND) under a Discovery Fund Seed Grant (R.J.W., J.P.C., and D.M.J.). N.S.L. is a fellow of the Chemistry-Biochemistry-Biology Interface (CBBI) Program at ND, supported by training grant T32GM075762 from the National Institute of General Medical Sciences. This content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. L.M.S. acknowledges support from the Arthur J. Schmitt Foundation and the Berry Family Foundation Graduate Fellowship through the Institute for Precision Health at ND. The DLS and ZP analyses were conducted at the Center for Environmental Science and Technology (CEST) at ND. J.P.C. and L.M.S. thank the ND Energy Materials Characterization Facility for the use of their XPS instrument and the ND Integrated Imaging Facility for assistance with the electron microscopy characterization of our nanoparticles. Last, L.M.S. gratefully acknowledges Joseph Liberko for creating the MatLab script used to analyze nanoparticle size distributions.

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