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Laboratory Experiment

Using NMR Spectroscopy To Measure Protein Binding Capacity on Gold Nanoparticles

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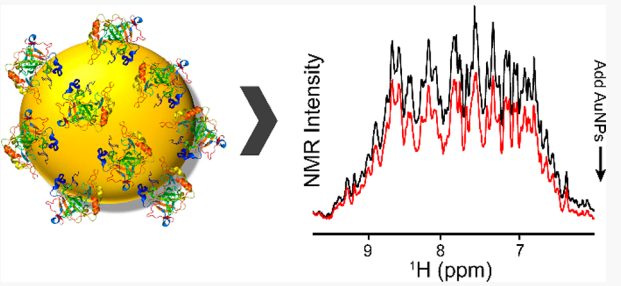
ABSTRACT: A simple one-dimensional ^{1}H NMR experiment that quantifies protein bound to gold nanoparticles has been developed for upper-division biochemistry and physical chemistry students. This laboratory experiment teaches the basics of NMR techniques, which is a highly effective tool in protein studies and supports students to understand the concepts of NMR spectroscopy and nanoparticle−protein interactions. Understanding the interactions of gold nanoparticles (AuNPs) with biological macromolecules is becoming increasingly important as interest in the clinical use of nanoparticles has been on the rise. Applications in drug delivery, biosensing, diagnostics, and enhanced imaging are all tangible possibilities with a better understanding of AuNP−protein interactions. The ability to use AuNPs as biosensors for drug delivery methods in cellular uptake is dependent on the amount of protein that is able to bind to the surface of the nanoparticle. This laboratory experiment solidifies concepts such as quantitative NMR spectroscopy while reinforcing precision laboratory titrations. Students learn how ^{1}H proton NMR spectra can be used to measure free protein in solution and protein bound to AuNPs. A simple formula is used to determine the binding capacity of the nanoparticle. This analysis helps students to understand the impact of nanoparticle−protein interactions, and it allows them to conceptualize macromolecular binding using NMR spectroscopy.



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INTRODUCTION

Research on nanoparticles has evolved into a major topic in chemistry.[1−3] Nanoparticles have decisively changed the field of medical research, where nanoparticles are used for drug delivery, biosensing, and imaging.[4,5] Among all other materials, gold nanoparticles (AuNPs) are observed to have lower toxicity when compared to silver, copper, and cobalt nanoparticles when used in the presence of different cell lines.[6−8] The low toxicity of AuNPs, combined with their tendency to spontaneously adsorb layers of protein to their surface, opens the door to many medical applications of nanoparticles. Small AuNPs (<10 nm) are known to be toxic when administered in the human body, and large AuNPs (>100 nm) are too large to be taken up by mammalian cells;[6,9] therefore, AuNPs for biomedical applications typically have diameters ranging from 15 to 100 nm.[10]

Despite the promise of beneficial applications, nanoparticles have not yet become common in clinical use, partially because little is known about the mechanisms that cause proteins and other biomaterials to spontaneously adsorb to the nanoparticle surface. In order to harness the full potential of nanoparticles, more must be learned about how proteins are able to bind to AuNPs.[11,12] By understanding more about the chemical principles controlling these interactions, it will be possible to predict more accurately the binding patterns and biological interactions of loaded nanoparticles when introduced into a living system.[13] Determining the quantity of protein that is able to bind to the surface of the AuNPs will allow for a better approximation of the amount of drug that can adsorb to the nanoparticles, as well.

As the field of nanoscience expands, there is an urgency for nanoscience education and emerging analytical techniques for high school students and undergraduates. Nanochemistry undergraduate laboratory experiments dedicated to nanoparticle−protein interactions are currently limited in scope.[14−22] Most of these have focused on spectroscopic characterization such as UV−vis spectroscopy to investigate the plasmonic properties of silver and gold nanoparticles.[15,17] There are also relatively few NMR spectroscopy-based undergraduate laboratories that focus on proteins.[23−27] Of

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these, most explore protein−small molecule interactions through enzyme kinetics[25] or small molecule binding.[23,24,26] The most advanced of these undergraduate NMR experiments provides students with a ^{13}C- and ^{15}N-labeled sample and explores contemporary NMR assignment strategies in an 11 week course.[27] None of these experiments, however, examines protein binding in the context of nanoparticles. Reported herein are AuNP-based upper-division undergraduate experimental procedures derived from recent research on quantification of protein binding to surfaces.[28,29] The underlying theories and measurement techniques behind these experiments are accessible to undergraduate students, and the modules furthermore allow students to learn cutting-edge methods in quantitative NMR spectroscopy.[30] Advanced NMR methods, such as dark-state exchange spectroscopy,[31] can be used to understand protein interactions on nanoparticles.[32] If a deeper study is desired, these approaches could be incorporated into the lab experiment, along with light scattering and other spectroscopic measurements.[30]

Learning objectives of this experiment include an introduction to both obtaining NMR spectra and interpreting spectra to understand nanoparticle−protein interactions. The exploration of NMR techniques and nanobioscience is relevant for students in introductory biochemistry or physical chemistry laboratories and lays out the foundation for characterization of nanoparticle−protein interaction using NMR instrumentation.[23,24,33] Students are tasked with characterizing the synthesized AuNPs and predicting the binding capacity (bound protein on one AuNP particle) of a protein for a particular size of AuNP.

EXPERIMENT

Module 1: Synthesis of 15 nm AuNPs

In the first experiment module of this lab, the citric acid reduction method is used to synthesize 15 nm gold nanoparticles.[34,35] The full experimental procedure for AuNP synthesis is given in the Supporting Information. Briefly, when preparing AuNPs, a 100 mL solution of 1 mM gold(III) chloride trihydrate is prepared using Milli-Q water and refluxed to its boiling point. Just as the solution begins to boil, 10 mL of 40 mM sodium citrate solution is added. The mixture is allowed to boil for 20 min before being removed from the heat. After being cooled to room temperature, the solution is divided into three 50 mL conical tubes and centrifuged at 9000 rpm (7700 rcf) for 45 min at 20 °C. The supernatant is then carefully removed, and the remaining nanoparticles are pooled and sonicated for 1 min intervals for a total of 6 min.

Module 2: Binding Capacity Measurements

BCA Protein Preparation. Lyophilized bovine carbonic anhydrase (BCA) is a commercially available metalloenzyme that catalyzes the interconversion between carbon dioxide and water and dissociates ions of carbonic acid. BCA was resuspended using Milli-Q water to prepare an approximate 0.1 g/mL solution that was used without further purification. The actual concentration of this solution was determined using UV−vis absorbance at 280 nm using a molar extinction coefficient of 50420 M^{-1} cm^{-1}.[36]

NMR Sample Preparation. All NMR samples were prepared with a total volume of 550 μL to ensure proper filling in a standard 5 mm NMR tube. Each sample was mixed in a 1.5 mL microcentrifuge tube, and all samples contained 20 μM of BCA, 40 mM phosphate buffer solution at a pH 6.6, 6% D_2O, and 200 μM DSS.[37] This can be done using stock solutions of 400 mM sodium phosphate (55 μL) and 5 mM DSS (22 μL), which can be prepared beforehand. Finally, Milli-Q water and AuNPs were added to the samples, with increasing concentrations of AuNPs. As the AuNP concentration was increased, the volume of added water was decreased, so that the total volume remained fixed at 550 μL. AuNPs were always added last, and microcentrifuge tubes were gently mixed by inversion. The value for the final AuNP concentration was typically 80 nM, which was prepared by adding a concentrated stock solution of 200 nM AuNPs. The samples were then left at room temperature for approximately 1 h to ensure the protein had ample time to bind to the AuNP surface.

Binding Capacity Measurement. ^1H NMR analysis was performed on each of the samples described above. The spectra gathered were then analyzed using the TopSpin software (Bruker Biospin, Billerica MA; available free of charge at the Bruker Website). After phasing, baseline correction, and normalizing all spectra to the DSS peak intensity, the difference in the intensity of the peaks in the amide proton region (6−10 ppm) was measured to calculate the amount of free protein in the samples. Due to the slow rotational diffusion of the nanoparticles, the bound proteins were not detected in the NMR spectrum; only the free protein remaining in the samples resulted in a signal in the amide region.

HAZARDS

Students should wear goggles, a lab coat, and gloves throughout the experiment. Aqua regia (nitric acid and hydrochloric acid) is highly caustic, and particular care should be exercised when washing glassware. Used aqua regia should be disposed of using the appropriate regulatory guidelines. A fume hood should be used for heating during the nanoparticle synthesis. The nanoparticles themselves are not hazardous in this preparation,[38] and they can be handled using standard laboratory chemical safety guidelines.

RESULTS AND DISCUSSION

In the present study, BCA was titrated with increasing concentration of the AuNPs. First, students successfully synthesized 15 nm AuNPs using citrate reduction method. The size of AuNPs is correlated to the ratio of sodium citrate to gold chloride used during synthesis. More concentrated sodium citrate produces smaller AuNPs and vice versa. This is because more citrate in the solution results in more nuclei formed at the beginning of the AuNP ripening process. As the amount of Au^{3+} precursor is constant, the average AuNP size is decreased with an increasing number of nuclei. The synthesized 15 nm AuNPs have a maximum plasmonic absorption peak at 520 nm (Figure 1).[39,40] Gold(III) chloride is initially yellow in color, and upon addition of sodium citrate, the solution becomes colorless and slowly turns to wine red as nanoparticles are synthesized (Figure 1, inset). The calculated extinction coefficient for 15 nm AuNP is 3.9×10^8 M^{-1} cm^{-1} at 520 nm.[41] Depending on how much supernatant is removed, the final AuNP concentration can range from 100 to 200 nM.

Once the AuNPs and BCA protein were made properly, the NMR sample preparation was carried out according to the steps previously discussed. The UV−vis peak was observed to shift as proteins and AuNPs were mixed (Figure 1). When

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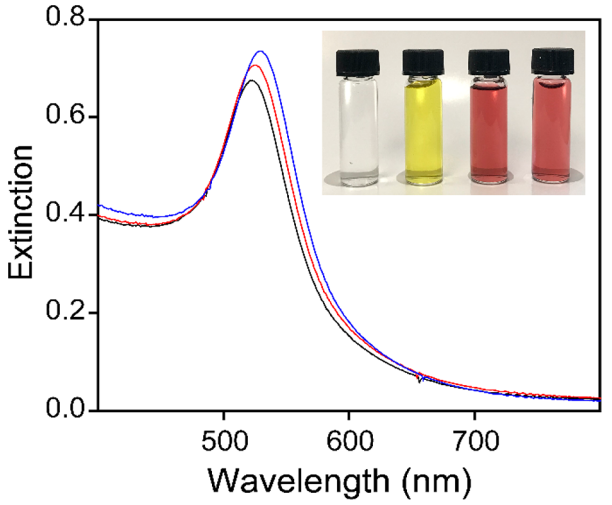

Figure 1. Initial synthesis of nanoparticle solutions. The inset shows the color change observed when AuNPs are synthesized from citric acid and gold(III) chloride trihydrate. From left to right, the vials are 40 mM sodium citrate, 1 mM $HAuCl_4$, 2 nM 15 nm AuNPs, and 2 nM AuNPs in the presence of 20 μM BCA. The main graph shows the UV−visible spectra of spherical 15 nm AuNPs in the absence and presence of BCA. The black curve represents the 15 nm AuNPs without protein at a nanoparticle concentration of 2 nM. The red curve represents the half saturation point when BCA is added, and the blue curve represents the fully saturated state. The red and blue curves are not collected as part of the experiment module, but they are shown to support the color change present in the inset.

comparing the ^{1}H NMR spectrum collected for each sample, it can be seen that, as the nanoparticle concentration increases, the intensities of the peaks in the amide proton region decrease as protein adsorbs to the nanoparticle surface.[28,30] An example spectra for BCA is shown for several concentrations of AuNPs in solution (Figure 2A).

TopSpin software was used to measure the ratio between the peaks within the amide region before and after addition of AuNPs. Relative to the sample containing no nanoparticles, this ratio (r) represents the fraction of signal remaining in solution after addition of AuNPs. Because the total concentration of protein is known (C_P), the free protein concentration is $C_F = rC_P$, and the bound protein concentration is $C_B = (1 - r)C_P$. The binding capacity for BCA is simply the number of bound BCA molecules per nanoparticle (eq 1):

$$N_{BCA} = \frac{C_B}{C_{NP}} = (1 - r)\frac{C_P}{C_{NP}} \quad (1)$$

where N_{BCA} is the binding capacity of the protein (number of BCA molecules per nanoparticle) and C_{NP} is the total concentration of AuNPs (in units of molarity). An alternative approach is possible if multiple experiments are performed. In this case, the binding capacity can be estimated by measuring the slope of a line when C_B is plotted against C_{NP} (Figure 2B). This approach requires binding to be tight (with a dissociation constant $K_d < 1$ μM),[28] and no intercept should be used in the linear fit.

The experimentally determined binding capacity was then compared to the predicted number of proteins on the AuNP surface, making two assumptions: First, it is assumed that proteins form a monolayer on the AuNP surfaces, and second, it is assumed that the proteins remain folded when bound. For

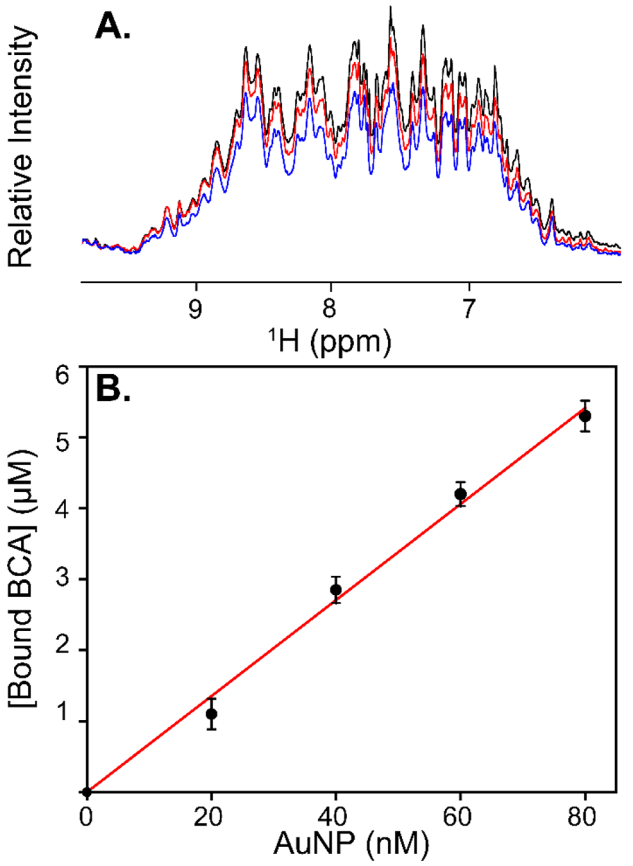

Figure 2. Student-collected example of overlaid ^{1}H NMR spectra of BCA as the concentration of 15 nm AuNPs is increased. (A) Amide proton NMR region of BCA in the presence of 0 (black), 20 (red), and 60 (blue) nM AuNPs. The amide peak intensities are reduced as AuNP concentration is increased, a result of protein binding. (B) Concentration of bound protein was plotted against various concentrations of AuNP, and the best-fit line (red) represents the binding capacity of BCA on 15 nm AuNP. Whereas multiple points are plotted here, a simple two-point determination is described in the laboratory procedure.

BCA, the folded state radius of gyration (R_G) is 1.7 nm.[42] Therefore, one single molecule of BCA is predicted to occlude $SA_{BCA} = \pi R_G^2 = 9.1$ nm^2 of surface area (SA) when bound to a surface. A 15 nm AuNP has a total surface area of $SA_{NP} = 4\pi R_{NP}^2 = 707$ nm^2. Therefore, the number of monolayer and folded BCA molecules predicted to bind the AuNP surface can be determined using eq 2:[28]

$$N_{BCA} = \frac{SA_{NP}}{SA_{BCA}} = 4\left(\frac{R_{NP}}{R_G}\right)^2 = 76 \quad (2)$$

Student-generated results for BCA (using several different AuNP concentrations) are shown in Figure 2B. In these experiments, the experimental binding capacity (N_{BCA}) was determined to be 68.

■ ASSESSMENT OF STUDENT LEARNING OBJECTIVES

This upper-division undergraduate experiment was performed as an introductory biochemistry laboratory experiment to four different sections of students (20 students in the pilot, along with 16 in two other classes, or 36 students total) in a physical chemistry course. The learning objectives for this experiment

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are as follows: (1) Students should gain exposure into the principles that drive the rapidly expanding field of nanotechnology. (2) Students should understand the experimental and theoretical background of NMR spectroscopy applied to protein-nanoparticle binding.

Of the students who participated in this experiment, 85% were able to successfully synthesize 15 nm AuNPs and calculate the BCA concentration. In addition, 80% of students were able to determine an accurate theoretical binding capacity for BCA on 15 nm AuNPs. Eighty percent of students correctly calculated the experimental binding capacity using NMR spectra, including the calculation of water suppression duration in the pulse program. Ninety-four percent of the students could correctly describe why a discrepancy might occur between the theoretical and experimental binding capacity. Student evaluation of the laboratory experiment indicated that they felt this topic "engaged their interest" (average of 4.6 "agree" on a Likert scale of 1−5) and they would "recommend this lab to others" (4.6 out of 5).

This two-part experiment allows students to synthesize nanoparticles as well as to learn the techniques used for the acquisition and analysis of quantitative NMR spectroscopy results. This builds on the organic chemistry curriculum by reinforcing that NMR spectroscopy is a quantitative analytical tool in addition to a method for characterizing reaction products. The students also have an opportunity to practice basic chemistry skills such as solution preparation and titrations. Students finish the experiment with an increased knowledge in the field of nanotechnology, and they understand how different analytical techniques can be valuable when studying nanoparticle−protein interactions. NMR spectroscopy is a commonly used analytical tool that is applicable in many different fields of research, and knowledge of NMR spectroscopy benefits the students as they pursue careers in the sciences. General exposure to nanoparticle−protein interactions also gives students a greater appreciation for the growing field of nanotechnologies. This experiment therefore provides an opportunity for students to delve into this exciting topic of research to introduce the possibility of a career in nanotechnology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.9b00625.

Detailed protocol on the synthesis of 15 nm AuNPs, instructions for measuring binding capacity using NMR, a student worksheet, instructor's notes, and a list of all reagents and equipment needed for the experiment (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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