Characterizing Single Polymeric and Protein Nanoparticles with Surface Plasmon Resonance Imaging Measurements

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ABSTRACT: Near-infrared surface plasmon resonance imaging (SPRI) microscopy is used to detect and characterize the adsorption of single polymeric and protein nanoparticles (PPNPs) onto chemically modified gold thin films in real time. The single-nanoparticle SPRI responses, Δ%RNP, from several hundred adsorbed nanoparticles are collected in a single SPRI adsorption measurement. Analysis of Δ%RNP frequency distribution histograms is used to provide information on the size, material content, and interparticle interactions of the PPNPs. Examples include the measurement of log-normal Δ%RNP distributions for mixtures of polystyrene nanoparticles, the quantitation of bioaffinity uptake into and aggregation of porous NIPAm-based (N-isopropylacrylamide) hydrogel nanoparticles specifically engineered to bind peptides and proteins, and the characterization of the negative single-nanoparticle SPRI response and log-normal Δ%RNP distributions obtained for three different types of genetically encoded gas-filled protein nanostructures derived from bacteria.

KEYWORDS: surface plasmon polaritons, single-nanoparticle refractive index, NIPAm-based hydrogel nanoparticle, melittin, concanavalin A, gas vesicle, protein nanostructure

The rational design, synthesis, and characterization of both polymeric and protein nanoparticles (PPNPs) for applications in materials, catalysis, and biotechnology have become a significant component of the current nanoscience revolution. PPNPs have been constructed from a wide variety of polymeric materials including single-chain or cross-linked polymers, dendrimers, synthetic polypeptides, proteins, and polysaccharides.1−6 PPNPS can be designed to form compact structures, porous hydrogels, or other three-dimensional structures that can exhibit a wide variety of rheological properties, display a large number of interfacial chemical moieties with specific affinities or reactivities on the outside of the nanoparticle, or incorporate internal chemical binding sites that can be used to capture and release chemical or smaller nanoparticles.7−10 Examples include elastin-like polypeptide nanoparticles that are biodegradable and thermally responsive,11,12 polysaccharide-based nanoparticles for medical diagnostics and therapies,13 and cross-linked N-isopropylacrylamide (NIPAm) hydrogel nanoparticles that incorporate a mixture of chemical functional groups to create specific binding sites for bioaffinity uptake.14−16 Genetically coded protein nanostructures with acoustic properties, such as gas vesicles (GVs), have been identified for use as ultrasound and magnetic resonance imaging contrast agents.17−20

The characterization of PPNPs at the single-nanoparticle level is challenging. Unlike metallic or semiconductor particles, which often exhibit a strong size-dependent optical response,21−23 PPNPs typically do not possess any convenient spectroscopic markers. Additionally, PPNPs often contain a significant amount of solvent, and their size and composition may vary with external pH, temperature, or pressure. A particularly important but difficult measurement is the quantification of bioaffinity adsorption and uptake into single PPNPs that have been designed for drug delivery or toxin neutralization applications. PPNPs are typically characterized with a combination of bulk dynamic light scattering (DLS) and multiangle light scattering (MALS) measurements,24−26 cryo-TEM,27−28 and, if the PPNPs are sufficiently rigid, scanning probe measurements.29,30 In some studies, the incorporation of fluorophores into the nanoparticle has been employed to facilitate single-nanoparticle detection and to provide some limited characterization information.31,32

The optical technique of single-nanoparticle surface plasmon resonance imaging (SPIR) microscopy has recently emerged as
an excellent in situ refractive-index based method for the detection and characterization of single PPNPs. As first identified in 2010 by Zybina and Tao,33–35 an adsorbed nanoparticle can interact with traveling surface plasmon polariton waves created on a gold thin film surface to create a point diffraction pattern in the differential SPRI image. Single metallic nanoparticles, polymer nanoparticles, liposomes, cells, and viruses have been detected with SPRI microscopy.

The intensity of the diffraction pattern depends on the integrated refractive index of the nanoparticle and, thus, varies with nanoparticle size and material content. Real-time SPRI measurements have been used previously for the digital biosensing of single-nanoparticle bioaffinity adsorption events at chemically modified gold thin films.36–46 In addition to nanoparticle-counting measurements, changes in the intensity of the average single-nanoparticle SPRI response (⟨Δ%RNP⟩) have been used to quantitate the bioaffinity uptake of polypeptides and proteins by hydrogel nanoparticles.32,43

Determining the distribution of Δ%RNP values obtained during a single-nanoparticle SPRI adsorption measurement in addition to the average response can provide much more detailed information about a population of PPNPs. Since the Δ%RNP response depends on the integrated refractive index of the nanoparticle, Δ%RNP frequency distributions will reflect variations in both nanoparticle size and composition. An example of the latter would be changes in a Δ%RNP distribution created by variations in molecular uptake into a population of PPNPs designed for drug delivery. While ensemble measurements such as DLS can provide limited information on the moments of a PPNP nanoparticle distribution, single-nanoparticle SPRI measurements can directly measure the detailed frequency distribution histogram of a PPNP population.

In this paper, we provide three different examples of how to obtain and use single PPNP Δ%RNP distributions from real-time SPRI adsorption measurements. As a first case, we demonstrate that Δ%RNP distributions can be used to measure nanoparticle size distributions for mixtures of solid polystyrene (PS) nanoparticles. In a second set of experiments, we demonstrate how Δ%RNP distributions obtained from porous NIPAm-based hydrogel nanoparticles (HNPs) can be used to monitor changes in PPNP structure and aggregation due to the bioaffinity uptake of peptides and proteins. In the final example, we show that the adsorption of gas-filled protein nanostructures produces an unusual negative single-nanoparticle SPRI response with a Δ%RNP distribution that depends on the shape and size of the particle. The three examples presented in this paper have been chosen to demonstrate that the single-nanoparticle SPRI measurements can be applied to three very different classes of PPNPs: solid polymer nanoparticles, highly porous, solvent-swollen polymer nanoparticles, and protein nanostructures that enclose a gas volume.

RESULTS AND DISCUSSION

Single-Nanoparticle SPRI Adsorption Measurements.

The detection and characterization of single polymer and protein nanoparticles was achieved by using real-time SPRI microscopy measurements to detect the irreversible adsorption of individual nanoparticles onto a chemically modified gold thin film surface. The optical setup of the near-infrared single-nanoparticle SPRI microscope used in these experiments is shown in Figure 1a and has been described in detail in a previous publication.31 Briefly, an 814 nm laser was expanded, collimated, and then polarized before being directed off-axis through the back of a high numerical aperture microscope objective and onto the back of a gold-coated glass coverslip. The reflected image was captured with a CMOS camera. A nanoparticle solution was exposed to the top of the gold-coated glass coverslip immediately preceding the image acquisition process. (b) A point diffraction pattern is observed in the SPRI differential reflectivity image when a 170 nm polystyrene (PS) nanoparticle adsors to the chemically modified gold surface. (c) Quantitative map displaying the Δ%R pixel intensities for the single-nanoparticle point diffraction pattern in (b). A sharp spike in Δ%R intensity is observed at the center of the diffraction pattern (the intersection of the two white dotted lines). We define Δ%RNP as the average of the Δ%R values for the nine pixels at and surrounding the pixel with the maximum Δ%R intensity. (d) Δ%RNP frequency distribution histogram obtained from the SPRI adsorption measurement of 170 nm PS nanoparticles. The average Δ%RNP value for this experiment was 2.19 ± 0.05% and is plotted in the figure as a black dotted line. The Δ%RNP distribution is also fit to a probability density function (PDF) with location (μ) and scale (σ) parameters of 0.76 and 0.21, respectively.

Figure 1. (a) Schematic diagram of the SPRI microscope. A gold-coated knife-edge mirror was used to direct collimated p-polarized light off-axis through the microscope objective and onto the back of the gold-coated glass coverslip. The reflected image was captured with a CMOS camera. A nanoparticle solution was exposed to the top of the gold-coated glass coverslip immediately preceding the image acquisition process. (b) A point diffraction pattern is observed in the SPRI differential reflectivity image when a 170 nm polystyrene (PS) nanoparticle adsors to the chemically modified gold surface. (c) Quantitative map displaying the Δ%R pixel intensities for the single-nanoparticle point diffraction pattern in (b). A sharp spike in Δ%R intensity is observed at the center of the diffraction pattern (the intersection of the two white dotted lines). We define Δ%RNP as the average of the Δ%R values for the nine pixels at and surrounding the pixel with the maximum Δ%R intensity. (d) Δ%RNP frequency distribution histogram obtained from the SPRI adsorption measurement of 170 nm PS nanoparticles. The average Δ%RNP value for this experiment was 2.19 ± 0.05% and is plotted in the figure as a black dotted line. The Δ%RNP distribution is also fit to a probability density function (PDF) with location (μ) and scale (σ) parameters of 0.76 and 0.21, respectively.
The center of each nanoparticle diffraction pattern has a sharp ∆%R (change in percent reflectivity) maximum that can be used to quantify the intensity of the single-nanoparticle SPRI response. Figure 1c shows a quantitative map of the ∆%R pixel intensities for a typical single-nanoparticle SPRI diffraction pattern. As described previously, the average of the nine ∆%R pixel intensities (a 3 × 3 array) at and surrounding the maximum is used to calculate the single-nanoparticle SPRI refraction reflectivity response that we denote as ∆%RNP. The single-nanoparticle SPRI diffraction pattern has been described previously by several researchers as the sum of a traveling plane wave and a spherical wave. Using the average values of the 3 × 3 array of nine pixels around the maximum is a simple, yet effective method to improve the spatial resolution of the nanoparticle location on the surface. For this experiment, two PS nanoparticles irreversibly adsorbed onto the MUAM surface. An example of a ∆%RNP frequency distribution histogram obtained from an in situ real-time SPRI adsorption measurement of 170 nm PS nanoparticles onto a chemically modified gold thin film is shown in Figure 1d (details of this experiment are given in the next section). The ∆%RNP for this measurement is also plotted in Figure 1d as a black dotted line. It is evident from the distribution that the ∆%RNP values are not symmetrically distributed about ⟨∆%RNP⟩. Therefore, in order to more precisely quantify this distribution, in addition to a standard deviation (s), we calculate a skewness (g) from the set of ∆%RNP values, where the skewness is proportional to the third central moment m3.

\[
g = \frac{m_3}{s^3} = \frac{1}{n} \sum \left( \Delta \% R_{NP} - \langle \Delta \% R_{NP} \rangle \right)^3
\]

The skewness can be either positive or negative, depending on which side of ⟨∆%RNP⟩ the distribution is skewed; for the data in Figure 1d, g = 0.68.

The ∆%RNP distribution of 170 nm PS nanoparticles is also fitted to a log-normal probability density function (PDF), described as

\[
\text{PDF} = \frac{1}{\Delta \% R_NP \sigma \sqrt{2\pi}} \exp \left[ -\frac{\left( \ln(\Delta \% R_{NP}) - \mu \right)^2}{2\sigma^2} \right]
\]

where \( \mu \) and \( \sigma \) are the location and scale parameters, respectively. This log-normal fit is plotted in Figure 1d as a black solid line, and it is apparent that a log-normal probability density function gives an accurate fit of the data. Previous size measurements on PS nanoparticles have also followed a log-normal distribution.

Mixtures of Polystyrene Nanoparticles. As a first demonstration that single-nanoparticle SPRI measurements can provide useful information on polydisperse polymer nanoparticle samples, a series of single-nanoparticle SPRI adsorption measurements were performed on three solutions of carboxyl-terminated PS nanoparticles: 85 nm diameter PS nanoparticles, 170 nm diameter PS nanoparticles, and a one-to-one mixture of 85 and 170 nm PS nanoparticles. For each SPRI adsorption measurement, PS nanoparticle solutions were exposed to a gold surface modified with an amine-terminated (11-mercaptoundecamine, MUAM) self-assembled monolayer. SPRI reflectivity images were collected as the negatively charged carboxyl-terminated PS nanoparticles electrostatically and irreversibly adsorbed to the MUAM surface. An example SPRI differential reflectivity image from the sample of mixed size PS nanoparticles is shown in Figure 2a. As seen in the image, two PS nanoparticles irreversibly adsorbed onto the MUAM surface within the three-second time frame. The larger, more intense point diffraction pattern near the top of the image is attributed to the adsorption of a 170 nm PS nanoparticle, whereas the smaller, less intense point diffraction pattern near the bottom of the image is attributed to the adsorption of an 85 nm PS nanoparticle. The intensity of each nanoparticle point diffraction pattern is quantitated by calculating a ∆%RNP value as described in the previous section. For the two PS

| nanoparticle | diameter (nm) | standard deviation (nm) | (Δ% RNP) | standard deviation (s) | 95% CI | skewness (g) | μ | σ | no. of NNPs |
|--------------|---------------|-------------------------|----------|-------------------------|-------|--------------|---|---|------------|
| PS (A)       | 85            | 25                      | 0.34     | 0.10                    | 0.01  | 0.59         | −1.13 | 0.31 | 354        |
| PS (B)       | 170           | 50                      | 5.19     | 0.48                    | 0.05  | 0.68         | 0.76  | 0.21 | 365        |
| HNP          | 271           | 55                      | 1.67     | 0.43                    | 0.05  | 0.60         | 0.48  | 0.27 | 324        |
| HNP + 2 μM melittin | 272 | 65                      | 2.79     | 0.52                    | 0.08  | 0.02         | 1.01  | 0.20 | 172        |
| HNP          | 272           | 50                      | 0.90     | 0.27                    | 0.03  | 0.55         | −0.15 | 0.31 | 289        |
| HNP + 500 nM ConA | 357 | 75                      | 3.6      | 1.3                     | 0.2   | 0.79         | 1.22  | 0.37 | 307        |
| HNP + 500 nM ConA + 1 mM Man | 338 | 65                      | 2.04     | 0.60                    | 0.07  | 0.05         | 0.66  | 0.36 | 270        |
| HNP + 500 nM ConA + 10 mM Man | 320 | 55                      | 1.74     | 0.41                    | 0.05  | 0.30         | 0.53  | 0.24 | 241        |
| Mega GV      | −             | −                       | −0.49    | 0.26                    | 0.03  | −1.28        | −0.84 | 0.52 | 274        |
| Ana GV       | −             | −                       | −1.07    | 0.44                    | 0.04  | −1.53        | −0.0083 | 0.38 | 395        |
| Halo GV      | −             | −                       | −3.0     | 1.5                     | 0.2   | −0.74        | 0.95  | 0.58 | 345        |

*Log-normal PDF location parameter. Log-normal PDF scale parameter. Size measurements for GV are reported in Table 2.
Figure 2. (a) Example SPRI differential reflectivity image of a mixed sample of PS nanoparticles. The larger, more intense point diffraction pattern represents a 170 nm PS nanoparticle, and the smaller, less intense point diffraction pattern represents an 85 nm PS nanoparticle. The total image area is 58.5 μm × 58.5 μm. (b) Time-dependent distribution of Δ% RNP values for the mixture of 85 and 170 nm PS nanoparticles. Each blue circle represents the Δ% RNP values for a single PS nanoparticle irreversibly adsorbing to the chemically modified surface. The two red circles represent the Δ% RNP values for the point diffraction patterns in the differential reflectivity image (a) that adsorbed to the surface at the 225 s mark of the experiment (black dotted line). Δ% RNP frequency distribution histograms obtained from three different SPRI adsorption measurements of (c) 85 nm PS nanoparticles, (d) 170 nm PS nanoparticles, and (e) a one-to-one mixture of 85 and 170 nm PS nanoparticles. Average Δ% RNP values for each size of PS nanoparticle are plotted as a black dotted line. The average Δ% RNP values for 85 and 170 nm PS nanoparticles are 0.34 ± 0.01% and 2.19 ± 0.05%, respectively.

In Figure 2a, it is apparent from the histogram in Figure 2e that the distribution obtained from the mixed size PS nanoparticle sample is simply the sum of the two single-size PS nanoparticle distributions. The Δ% RNP values obtained for each size of PS nanoparticle are plotted in Figure 2e and are the same values as those obtained from the experiments in Figure 2c and d. These results unequivocally demonstrate that the single-nanoparticle SPRI measurements can be used to study polydisperse mixtures of nanoparticles. Using the data presented in Figure 2, we estimate that we can differentiate two populations of PS nanoparticles that have a difference in diameter greater than 40 nm.

Molecular Uptake into Hydrogel Nanoparticles and Aggregation of Hydrogel Nanoparticles. In a second set of experiments, we demonstrate that Δ% RNP frequency distribution histograms from single-nanoparticle SPRI measurements can be used to characterize the bioaffinity uptake of molecules into porous PPNPs, such as NIPAm-based HNPs. HNPs are solvent-swollen nanoparticles (up to ~65% solvent by volume as estimated from MALS measurements) that can be engineered to incorporate chemical moieties with specific affinity for various biomolecules. We have previously shown that (Δ% RNP) values from single-nanoparticle SPRI measurements can be used to study the uptake of the peptide melittin and the lectin concanavalin A (ConA) into specifically designed HNPs. In this paper, we demonstrate that the analysis of Δ% RNP frequency distribution histograms can be used to provide additional information on the uptake of these molecules into HNPs.

An example of a Δ% RNP frequency distribution histogram of peptide uptake by HNPs is shown in Figure 3. As depicted in Figure 3a, NIPAm-based HNPs (272 nm in diameter as measured by DLS) were synthesized with specific affinity for melittin, a small peptide composed of 26 amino acid residues. Single-nanoparticle SPRI measurements on these HNPs, in both the absence and presence of melittin, were used to quantify the Δ% RNP response. Plotted in Figure 3b are two Δ% RNP frequency distribution histograms: the Δ% RNP distribution for HNPs alone (transparent blue bars) and the Δ% RNP distribution for HNPs in the presence of 2 μM melittin (solid red bars). The two distributions in Figure 3b show that there is an overall increase in the average single-nanoparticle Δ% RNP response due to the uptake of melittin into the HNPs, which is an increase in the total integrated refractive index of the HNPs. Reported in Table 1 are the values for Δ% RNP, s, CI, g, μ, and σ obtained from the measurements. However, although there is an increase in Δ% RNP, there are no significant increases observed in the size or skewness of the Δ% RNP distributions of HNPs in the presence of melittin. Specifically, the value for σ decreases from 0.27 to 0.20 for HNPs in the presence of melittin, and the relative standard deviation (σ/Δ% RNP) also decreases (see Table 1). These results suggest that melittin uptake does not affect the structure...
of the HNPs, a conclusion that is corroborated with DLS measurements that show no change in the average hydrodynamic diameter for the hydrogels in the presence of 2 μM melittin (data also reported in Table 1).

In comparison, large changes in the Δ%RNP frequency distribution histograms were observed upon the uptake of the lectin ConA into HNPs modified with mannose. ConA is a large protein (MW = 104 kDa) with four subunits and a high binding specificity for mannose. Single-nanoparticle SPRI measurements were used to study the binding of ConA to mannose-modified HNPs as shown schematically in Figure 4a. Plotted in Figure 4b are two Δ%RNP frequency distribution histograms: mannose-modified HNPs only (transparent blue bars) and mannose-modified HNPs in the presence of 500 nM ConA (solid red bars). The ⟨Δ%RNP⟩, s, 95% CI, g, μ, and σ values for these two distributions are reported in Table 1. As evident from the data, not only is there an increase in (Δ%RNP) in the presence of ConA, but there is also a significant increase in the width of the Δ%RNP distribution. Specifically, there is 5-fold increase in the standard deviation of the Δ%RNP distribution for mannose-modified HNPs in the presence of ConA. Additionally, we observe an increase in the skewness and scale parameter. Because ConA has the capability to bind to multiple mannoses, ConA can induce aggregation of the mannose-modified HNPs by cross-linking. We attribute the changes in the Δ%RNP distributions to the aggregation of the mannose-modified HNPs induced by interparticle interactions of ConA that is bound to the outer regions of the HNPs. These results are also confirmed with DLS, which shows an increase in average hydrodynamic diameter of the mannose-modified HNPs from 272 to 357 nm.

To further study ConA binding to mannose-modified HNPs, additional single-nanoparticle SPRI measurements were made on the mixtures of mannose-modified HNPs and 500 nM ConA in the presence of free mannose in solution. By introducing free mannose into solution, we can induce competition between ConA binding to free mannose and mannose-modified HNPs and subsequently decrease the ConA-induced aggregation of mannose-modified HNPs. The Δ%RNP frequency distribution histograms for single-nanoparticle SPRI measurements of mannose-modified HNPs and 500 nM ConA with the addition of 1 mM mannose (solid green bars) and 10 mM mannose (solid orange bars) are shown in Figure 4c and d, respectively. The ⟨Δ%RNP⟩, s, 95% CI, g, μ, and σ values for these distributions are also reported in Table 1. As in Figure 4b, the Δ%RNP frequency distribution for mannose-modified HNPs without ConA is also plotted in Figure 4c and d for comparison (transparent blue bars). The distributions plotted in Figure 4c and d clearly show increases in the both ⟨Δ%RNP⟩ and the width of the distributions, compared to measurements of mannose-modified HNPs without ConA; however, these increases in ⟨Δ%RNP⟩ and the width of the distributions are less compared to measurements of mannose-modified HNPs and 500 nM ConA but without free mannose in solution (Figure 4b). This observation can also be seen quantitatively from the values listed in Table 1. For example, the standard deviation for mannose-modified HNPs increases by 480%, 220%, and 150% in the presence of 500 nM ConA and 0, 1, and 10 mM mannose, respectively. The Kd for ConA binding to...
monovalent mannose is on the order of $10^{-3}$ to $10^{-4}$ M. The observation that 10 mM monovalent mannose did not eliminate ConA interactions with the mannose-modified HNPs implies that there is a strong binding affinity between ConA and mannose-modified HNPs. It is well reported that the strength of interactions between sugars and lectins can be enhanced via multivalent binding, and various two- and three-dimensional sugar–polymer networks enhance the potency of the sugar–lectin interactions. It has been previously demonstrated that mannose-modified HNPs have $K_d$ values in the micromolar to nanomolar range.

**Gas Vesicle Protein Nanostructures.** As a final example of the utility of single-nanoparticle SPRI measurements of PPNPs, we demonstrate the use of single-nanoparticle SPRI measurements to characterize gas vesicle protein nanostructures. GVs are hollow gas-filled bacterial protein nanostructures composed of a ~2 nm protein shell that excludes water but allows gas to diffuse in and out of the particle. In this work, we characterized three genotypes of GVs encoded by the bacteria *Bacillus megaterium* (Mega GVs), *Anabaena flos-aquae* (Ana GVs), and *Halobacterium salinarum* (Halo GVs). TEM images of the three varieties of GVs are displayed in Figure 5a, and a schematic illustration of an Ana GV is shown in Figure 5b. The preparation of these GVs has been reported previously. The observation that 10 mM monovalent mannose did not eliminate ConA interactions with the mannose-modified HNPs implies that there is a strong binding affinity between ConA and mannose-modified HNPs. It is well reported that the strength of interactions between sugars and lectins can be enhanced via multivalent binding, and various two- and three-dimensional sugar–polymer networks enhance the potency of the sugar–lectin interactions. It has been previously demonstrated that mannose-modified HNPs have $K_d$ values in the micromolar to nanomolar range.

**Table 2. Size Measurements from TEM and Volume, Molecular Weight, and Gas-to-Protein Ratio Calculations for Gas Vesicles**

| nanostructure | Mega GV | Ana GV | Halo GV |
|---------------|---------|--------|---------|
| length (nm)   | 249     | 519    | 400     |
| $s_1$ (nm)    | 99      | 160    | 113     |
| 95% CI (nm)   | 25      | 31     | 20      |
| diameter (nm) | 73      | 136    | 251     |
| $s_2$ (nm)    | 14      | 21     | 51      |
| 95% CI (nm)   | 4       | 4      | 9       |
| volume (nm³)  | $7.4 \times 10^3$ | $6.4 \times 10^4$ | $6.6 \times 10^6$ |
| $s_3$ (nm³)   | $0.8 \times 10^3$ | $0.4 \times 10^4$ | $0.4 \times 10^6$ |
| 95% CI (nm³)  | $2 \times 10^3$ | $8 \times 10^4$ | $7 \times 10^6$ |
| # of GVs      | 61      | 107    | 125     |
| estimated GV molecular weight (MDa) | 72 | 320 | 282 |
| estimated gas-to-protein volume ratio | 8 | 16 | 19 |

We have previously observed both positive and negative diffraction patterns for PS, hydrogel, and other nanoparticles due to the transient adsorption and subsequent desorption of nanoparticles for the case where nanoparticles are not irreversibly adsorbed onto the chemically modified gold thin film. The observed negative diffraction pattern due to desorption always occurred after and at the same location as the previous positive diffraction pattern. For the positively charged MUAM-modified gold thin film, the GVs are irreversibly adsorbed, and the adsorption event always created a negative diffraction pattern. Occasionally, we did observe most dramatically in Figure 5d, which quantifies a sharp negative spike in $\Delta R$ that is observed at the center of the point diffraction pattern (intersection of the two black dotted lines). Calculation of $\Delta R_{NP}$ for an individual GV results in a negative value. Because the volumes of GVs are primarily composed of air, the displacement of water ($n_{\text{water}} = 1.33$, where $n$ is the refractive index) with the GV ($n_{\text{air}} = 1.0$) causes a decrease in the local refractive index at the location of the GV adsorption and consequently yields a negative $\Delta R_{NP}$ value.

Figure 5. (a) TEM images of the three genotypes of gas vesicle (GV) nanostructures: Halo GVs (left), Ana GVs (middle), and Mega GVs (right). (b) GVs are composed of a ~2 nm protein shell that excludes water but allows gas to flow in and out of the particle. (c) A negative point diffraction pattern is observed in the SPRI differential reflectivity images when a GV electrostatically adsorbs to the chemically modified gold surface. (d) Quantitative map displaying the $\Delta R$ pixel intensities for the single-GV point diffraction pattern. A sharp, negative spike in $\Delta R$ intensity is observed at the center of the diffraction pattern (the intersection of the two black dotted lines). We observe negative point diffractions for GV adsorption events due to the decrease in interfacial refractive index from water to air (GV).
positive diffraction patterns, which we attribute to the desorption of GVs, but this occurred less than 5% of the time.

The $\Delta R_{NP}$ frequency distribution histograms for single-nanoparticle SPRI adsorption measurements of all three types of GVs are displayed in Figure 6: Mega GVs (Figure 6a), Ana GVs (Figure 6b), and Halo GVs (Figure 6c). Similar to the PS nanoparticles, the absolute $\Delta R_{NP}$ values for all three types of GVs follow log-normal distributions. The values of $\langle \Delta R_{NP} \rangle$, 5% CL, $\mu$, and $\sigma$ for the GVs are all reported in Table 1. All of the GVs have larger relative standard deviations ($\sigma/\langle \Delta R_{NP} \rangle$), skew factors ($\gamma$), and log-normal scale factors ($\sigma$) as compared to PS nanoparticles (Table 1). We attribute these larger log-normal distributions to the heterogeneous nature of the GV biosynthesis. As expected, $\langle \Delta R_{NP} \rangle$ values for the three types of GVs increase as the total volume of the GV increases (in the order Halo GV $>$ Ana GV $>$ Mega GV). However, a quantitative relationship of $\langle \Delta R_{NP} \rangle$ to GV volume is complex; the protein component of the GV makes a positive contribution to $\Delta R_{NP}$ while the gas volume makes a negative contribution. As seen in Table 2, the gas volume dominates over the protein volume in all the GVs, which is why we observe negative $\Delta R_{NP}$ values for all GVs. Moreover, the Ana and Mega GVs have a high length-to-width aspect ratio, which could alter the single-nanoparticle SPRI response. Because the GVs adsorbed to the surface from a quiescent 10 $\mu$L solution, we do not expect that there are any preferential orientations of the anisotropic GVs relative to the direction of the surface plasmon polaritons. The future incorporation of a microfluidic flow system for nanoparticle delivery to the gold surface could potentially be used to create oriented adsorbed GV populations. Since near-infrared surface plasmon polaritons have a decay length of approximately 200–300 nm perpendicular to the gold surface, Ana and Mega GVs that adsorb with their length perpendicular to the surface may fall outside the range of the surface plasmon polaritons and produce a smaller than expected $\Delta R_{NP}$.

**CONCLUSIONS**

In summary, the experiments presented in this paper have demonstrated that both the average single-nanoparticle response ($\langle \Delta R_{NP} \rangle$) and $\Delta R_{NP}$ frequency distribution measurements obtained from single-nanoparticle SPRI adsorption measurements can provide detailed characterization information for a variety of solid, porous, and gas-filled PPNPs. The $\Delta R_{NP}$ frequency distribution histograms of PS nanoparticles showed that $\Delta R_{NP}$ depends on nanoparticle volume for solid nanoparticles. The changes of $\langle \Delta R_{NP} \rangle$ observed upon uptake of melittin into porous HNPs demonstrate that the single-nanoparticle SPRI measurements can also measure changes in the total material content of a nanoparticle. The ConA binding to mannose-modified HNPs indicates that both bioaffinity uptake and nanoparticle aggregation can be studied through the $\Delta R_{NP}$ frequency distribution histograms. Finally, the most striking evidence that single-nanoparticle SPRI experiments measure changes in interfacial refractive index due to nanoparticle adsorption is the negative point diffraction patterns and $\Delta R_{NP}$ values observed for the adsorption of gas vesicles, a type of gas-filled protein nanostructure.

An important parameter to ascertain for these single-nanoparticle SPRI measurements on PPNPs is how narrow of a $\Delta R_{NP}$ frequency distribution can be measured. Since every PPNP $\Delta R_{NP}$ distribution determined in this paper could be fit with a log-normal distribution, we can use the scale parameter $\sigma$ to define the normal distribution. The lowest scale parameter observed in these experiments is $\sim$0.2, and thus this number is our current experimental lower limit for what we can measure for $\Delta R_{NP}$ log-normal distributions. With additional theoretical modeling of the single-nanoparticle SPRI response and the development of more accurate methods of determining $\Delta R_{NP}$, we expect that this lower limit can be improved in the future.

**METHODS**

**Hydrogel Nanoparticle Synthesis.** N-Isopropylacrylamide (NIPAm), acrylic acid (AAc), sodium dodecyl sulfate (SDS), V-501, and ammonium persulfate (APS) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). N,N'-Methylenebis(acrylamide) (BIS) was obtained from Fluka (St. Louis, MO, USA). N-tert-Butylacrylamide (TBAm) was obtained from Acros Organics (Geel, Belgium). NIPAm was recrystallized from hexane before use. All other chemicals were used as received.

HNPs for melittin uptake experiments were synthesized following the procedure detailed in Cho et al.42 The monomers NIPAm (53 mol %), TBAm (40 mol %), AAc (5 mol %), and BIS (2 mol %) were dissolved in 50 mL of nanopure water in a round-bottom flask for a total monomer concentration of 65 mM. TBAm was dissolved in 1 mL of ethanol before addition to the monomer solution. The surfactant SDS (1.7 mg) was also added to the monomer solution to control nanoparticle size. Nitrogen gas was bubbled through the solution for 30 min. Following the addition of a 0.5 $\mu$L aqueous solution containing 30 mg of APS, the polymerization was carried out in an oil bath preset to 60 $^\circ$C for 3 h under a nitrogen atmosphere. The
Solutions of PS nanoparticles were diluted in nanopure water to USA). Andor Neo sCMOS camera (South Windsor, CT, USA) by silicone isolation wells (Electron Microscopy Sciences, Hat C11 solution for 12 h. All Au surfaces were partitioned using adhesive immobilized with a hydrophobic 1-undecanethiol monolayer (C11, USA) by immersing the Au substrate in a 1 mM ethanolic MUAM coating with a 1 nm Cr adhesion layer and 45 nm Au. For PS borosilicate No. 1.5 coverslips (Fisherbrand, Pittsburgh, PA, USA) purchased from Polysciences, Inc. (Warrington, PA, USA). Au slides styrene spheres with mean diameters of 85 and 170 nm were coated with a 1 nm Cr adhesion layer and 45 nm Au. For PS nanoparticles and GV measurements, Au surfaces were immobilized by dialysis using 12 kDa dialysis membrane against an excess amount of nanopure water (changed more than three times a day) for 4 days. Mega GVs, which are natively clustered after puriﬁed from E. coli, as described previously.60 Briefly, cells were cultured to conﬂuency (and, in the case of E. coli, induced to express GVs) and lysed using hypertonic, hypotonic, or detergent lysis. GVs were isolated using centrifuigally assisted buoyancy puriﬁcation, and their concentration was measured using optical density at 500 nm. Mega GVs, which are natively clustered after puriﬁcation from bacteria, were unclustered with a solution of 6 M urea and 20 mM Tris-HCl (pH = 8.0), followed by two rounds of centrifugally assisted buoyancy puriﬁcation and overnight dialysis in 1X phosphate-buffered saline (PBS) (11.9 mM phosphates, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4, Fisher), before optical density quantiﬁcation and use in SPRI experiments. Transmission electron microscopy was performed on a Philips Tecnai T12 LaB6 120 kV after staining with 2% uranyl acetate.61 GV measurements were chemically modiﬁed with C11, and isolation wells were ﬁlled with 10 μL of 1× PBS to protect the C11 layer. For melittin uptake measurements, melittin (Sigma-Aldrich) was dissolved in 1× PBS and diluted to a concentration of 18 μM. HNPs were diluted in 1X PBS to a ﬁnal concentration of 20 μg/mL, and 18 μM melittin was added with a ﬁnal concentration of 2 μM. The HNP and melittin mixture was allowed to incubate at room temperature for 30 min before SPRI experiments. For ConA uptake measurements, mannose-modiﬁed HNPs were diluted in 1X PBS to a ﬁnal concentration of 20 μg/mL after mixing with ConA (Sigma-Aldrich) at a ﬁnal concentration of 500 nM. For mannose-modiﬁed HNP experiments with free mannose, α-(+)-mannose (Sigma-Aldrich) was also added to the solution at the speciﬁed concentration from a more concentrated solution in 1X PBS. The mannose-modiﬁed HNP and ConA mixtures were allowed to incubate at room temperature for a minimum of 30 min before SPRI experiments. Gas Vesicle SPRI Measurements. Au slides for GV measurements were chemically modiﬁed with MUAM, and isolation wells were ﬁlled with 10 μL of 1X PBS to protect the MUAM layer. The optical density at 500 nm was measured using a Nanodrop 2000 (Thermo Scientiﬁc). All GVs were diluted in 1X PBS to the concentrations speciﬁed for SPRI experiments: Mega GVs diluted to 1 nM, Ana GVs diluted to 10 nM, and Halo GVs diluted to 5 μM. Dynamic Light Scattering Measurements. The hydrodynamic diameters of PS nanoparticles were measured in aqueous solutions at 25 °C, and the hydrodynamic diameters of hydrogel nanoparticles were measured in 1X PBS at 25 °C by a DLS instrument equipped with Zetasizer software (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, U.K.). The authors declare no competing ﬁnancial interest. This work was supported by the National Science Foundation through grant CHE-1403506 (R.M.C.), the National Institutes of Health through grants R01-GM059622 (R.M.C.) and R01-EB018975 (M.G.S.), and the Heritage Medical Research Institute (M.G.S.). DLS data were acquired at the Laser Spectroscopy Facility in the Department of Chemistry at UCI. The authors acknowledge Y. Terada for the synthesis of p-acrylamidophenyl-α-L-mannopyranoside. REFERENCES (1) Nayak, S.; Lyon, L. A. Soft Nanotechnology with Soft Nanoparticles. Angew. Chem., Int. Ed. 2005, 44, 7686–7708. (2) Rao, J. P.; Geckeler, K. E. Polymer Nanoparticles: Preparation Techniques and Size-Control Parameters. Prog. Polym. Sci. 2011, 36, 887–913. (3) Shi, X.; Thomas, T. P.; Myc, L. A.; Kotlyar, A.; Baker, J. R., Jr. Synthesis, Characterization, and Intracellular Uptake of Carboxyl-Terminated Poly(amidoamine) Dendrimer-Stabilized Iron Oxide Nanoparticles. Phys. Chem. Chem. Phys. 2007, 9, 5712–5720. (4) Callahan, D. J.; Liu, W.; Li, X.; Dreher, M. R.; Hassouneh, W.; Kim, M.; Marszalek, P.; Chilkoti, A. Triple Stimulus-Responsive

Optical Setup. The detailed description of the construction of the near-infrared single-nanoparticle SPRI microscope is described in a previous publication.7 The microscope was built into the frame of an IX51 inverted microscope (Olympus, Tokyo, Japan). A 1 mW, 814 nm diode laser (Melles Griot, Carlsbad, CA, USA) was expanded and collimated using a spatial filter (Newport, Corp., Newport Beach, CA, USA). The beam was then polarized and focused with a lens (f = 200 mm) onto the back focal plane of a 100×1.49 numerical aperture oil immersion objective (Olympus). The beam was directed upward near the edge of the objective by a gold-coated knife-edge mirror (Thorlabs, Newton, NJ, USA) that was mounted on an X–Y micrometer, in order to adjust the incident angle on the sample. The reﬂected light was allowed to pass out the other side of the objective and acquired by an Andor Neo sCMOS camera (South Windsor, CT, USA) by accumulating 30 11-bit, 0.1 s exposures. Substrate Preparation. Substrates for all SPRI experiments were borosilicate No. 1.5 coverslips (Fisherband, Pittsburgh, PA, USA) coated with a 1 nm Cr adhesion layer and 45 nm Au. For PS nanoparticles and GV measurements, Au surfaces were immobilized with a positively charged alkanethiol monolayer (11-mercaptoundecamine, Dojindo Molecular Technologies, Inc., Gaithersburg, MD, USA) by immersing the Au substrate in a 1 mM ethanolic MUAM solution for 12 h. For HNP measurements, Au surfaces were immobilized with a hydrophobic 1-undecanethiol monolayer (C11, Sigma-Aldrich) by immersing the Au substrate in a 1 mM ethanolic C11 solution for 12 h. All Au surfaces were partitioned using adhesive silicone isolation wells (Electron Microscopy Sciences, Hatﬁeld, PA, USA). Polystyrene Particle SPRI Measurements. Carboxylate polystyrene spheres with mean diameters of 85 and 170 nm were purchased from Polysciences, Inc. (Warrington, PA, USA). Au slides chemically modiﬁed with MUAM were prepared and isolation wells were ﬁlled with 10 μL of nanopure water to protect the MUAM layer. Solutions of PS nanoparticles were diluted in nanopure water to concentrations of ~10^9 particles/mL for all measurements. For all SPRI experiments, 10 μL of nanoparticle solution was pipetted into the isolation well immediately preceding the image acquisition process. Hydrogel SPRI Measurements. Au slides for all hydrogel nanoparticle SPRI measurements were chemically modiﬁed with C11, and isolation wells were ﬁlled with 10 μL of 1× PBS to protect the C11 layer. For melittin uptake measurements, melittin (Sigma-Aldrich) was dissolved in 1× PBS and diluted to a concentration of 18 μM. HNPs were diluted in 1X PBS to a ﬁnal concentration of 20 μg/mL, and 18 μM melittin was added with a ﬁnal concentration of 2 μM. The HNP and melittin mixture was allowed to incubate at room temperature for 30 min before SPRI experiments. For ConA uptake measurements, mannose-modiﬁed HNPs were diluted in 1X PBS to a ﬁnal concentration of 20 μg/mL after mixing with ConA (Sigma-Aldrich) at a ﬁnal concentration of 500 nM. For mannose-modiﬁed HNP experiments with free mannose, α-(+)-mannose (Sigma-Aldrich) was also added to the solution at the speciﬁed concentration from a more concentrated solution in 1X PBS. The mannose-modiﬁed HNP and ConA mixtures were allowed to incubate at room temperature for a minimum of 30 min before SPRI experiments. Gas Vesicle SPRI Measurements. Au slides for GV measurements were chemically modiﬁed with MUAM, and isolation wells were ﬁlled with 10 μL of 1X PBS to protect the MUAM layer. The optical density at 500 nm was measured using a Nanodrop 2000 (Thermo Scientiﬁc). All GVs were diluted in 1X PBS to the concentrations speciﬁed for SPRI experiments: Mega GVs diluted to 1 nM, Ana GVs diluted to 10 nM, and Halo GVs diluted to 5 μM. Dynamic Light Scattering Measurements. The hydrodynamic diameters of PS nanoparticles were measured in aqueous solutions at 25 ºC, and the hydrodynamic diameters of hydrogel nanoparticles were measured in 1X PBS at 25 ºC by a DLS instrument equipped with Zetasizer software (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, U.K.). The authors declare no competing ﬁnancial interest. This work was supported by the National Science Foundation through grant CHE-1403506 (R.M.C.), the National Institutes of Health through grants R01-GM059622 (R.M.C.) and R01-EB018975 (M.G.S.), and the Heritage Medical Research Institute (M.G.S.). DLS data were acquired at the Laser Spectroscopy Facility in the Department of Chemistry at UCI. The authors acknowledge Y. Terada for the synthesis of p-acrylamidophenyl-α-L-mannopyranoside.
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NOTE ADDED AFTER ASAP PUBLICATION

The version of this paper that was published ASAP July 12, 2017, contained an error in eq 1. The corrected version was reposted July 13, 2017.