Dual-Wavelength Surface Plasmon Resonance for Determining the Size and Concentration of Sub-Populations of Extracellular Vesicles

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ABSTRACT

Accurate concentration determination of sub-populations of extracellular vesicles (EVs), such as exosomes, is of importance both in the context of understanding their fundamental biological role and of potentially using them as disease biomarkers. In principle, this can be achieved by measuring the rate of diffusion-limited mass uptake to a sensor surface modified with a receptor designed to only bind the sub-population of interest. However, a significant error is introduced if the targeted EV sub-population has a size, and thus hydrodynamic diffusion coefficient, that differs from the mean size and diffusion coefficient of the whole EV population and/or if the EVs become deformed upon binding to the surface. We here demonstrate a new approach to determine the mean size (or effective film thickness) of bound nanoparticles in general, and EV sub-population carrying a marker of interest, in particular. The method is based on operating surface plasmon resonance simultaneously at two wavelengths with different sensing depths and using the ratio of the corresponding responses to extract the particle size on the surface. By estimating in this way the degree of deformation of adsorbed EVs, we markedly improved their bulk concentration determination and showed that EVs carrying the exosomal marker CD63 correspond to not more than around 10% of the EV sample.
INTRODUCTION

Cell-to-cell communication does not rely on soluble molecules alone, but also on the action of membrane-enveloped extracellular vesicles (EVs). Although overlooked for long, EVs have today been identified to function as key genetic, protein and lipid carriers in a variety of biological processes, related *e.g.* to the immune system\(^1\) but also to occur during cancer\(^2\) and neurodegenerative diseases\(^3\). As a consequence, EVs constitute a potential source of new diagnostic biomarkers.\(^4\) However, their low concentration, the low copy numbers of different lipids, proteins and nucleic acids found in cell-produced EVs, and their large diversity make it challenging to characterize the biological and physicochemical properties (*e.g.*, concentration and geometrical size) of different sub-populations. Herein, this challenge was addressed by combining information about the EV size distribution determined in bulk with measurements of time-resolved kinetics of attachment of a specific EV sub-population to a surface plasmon resonance (SPR) sensor modified with the corresponding antibodies and operated in the dual-wavelength mode. We focus on the EV sub-population carrying the tetraspanin membrane protein CD63 considered one of several markers for exosomes,\(^5\) a type of EVs of endosomal origin which hold promise as diagnostic markers for various health disorders\(^6\) and as cargo carriers in drug delivery\(^7\), gene therapy\(^8\) and vaccine\(^9\) applications.

SPR offers, in addition to accurate quantification of the mass of adsorbed species and determination of biomolecular interaction kinetics,\(^10\) a means to determine the bulk concentration of biomolecules\(^11\). This opportunity stems from the possibility to perform biomolecular binding experiments in such a way that the rate of binding is controlled globally by diffusion of the target molecules to the interface. In this case, the surface coverage is a function of the diffusion
constant, $D$, and the bulk concentration, $C$.\textsuperscript{11-12} With these two independent means to determine the surface coverage, \textit{i.e.} both from the magnitude of the measured SPR signal and the rate of its changes, the bulk concentration can be determined, since $D$ is usually known. While most other approaches employed to determine biomolecular concentrations (\textit{e.g.}, UV spectroscopy and colorimetric methods such as Lowry, BCA and Bradford assays\textsuperscript{13}) require carefully purified suspensions, the ability to functionalize SPR sensor surface to specifically capture analytes of interest makes it possible to directly determine the concentration of targeted biomolecules present in complex biological fluids\textsuperscript{14}. However, certain complications arise when this approach is used to determine the concentration of biological supramolecular assemblies with dimensions approaching or extending beyond the decay length, $\delta$, of the intensity of the exponentially vanishing SPR evanescent field, such as for, thick films\textsuperscript{15}, virus particles\textsuperscript{16-17}, drug-delivery vehicles\textsuperscript{18}, and EVs including exosomes\textsuperscript{19-20}. Although the conventional approach introduced by Jung et al.\textsuperscript{15} for interpretation of single-wavelength SPR measurements yields accurate results for certain cases, like thick films or cylindrically shaped islands, its applicability for more complex situations like supramolecular assemblies is still not straightforward. In our recent study\textsuperscript{20}, we extended the SPR formalism proposed by Jung et al.\textsuperscript{15} to describe spherical and deformed vesicles, allowing us to determine the bulk concentration of both synthetic lipid vesicles and CD63-positive extracellular vesicles (EVs) derived from human mast cells. Under the (unverified) assumption that \textit{all} EVs carried the biomarker CD63 used to ensure specific binding to the sensor surface, it was shown that the concentration determination was in fairly good agreement (within a factor of less than 2) with the concentration obtained from an independent determination of the total protein content. For synthetic lipid vesicles, an additional complication was identified, namely an overestimation of the bulk concentration that approached 50%, which
was likely due to adsorption-induced vesicle deformation, a phenomena that could also not be excluded for EVs.

The key elements of this analytical complications are summarized in Figure 1, which illustrates that the rate of binding to a surface is under diffusion-limited conditions, controlled by the (mean) diffusion constant, $D$, of the nanoparticles, which is in turn inversely proportional to their (mean) radius, $r_{\text{mean}}$. In the case where the size of adsorbed objects approaches the decay length, $\delta$, of the intensity of the evanescent SPR field, translation of the measured SPR response into bound mass, requires that the film thickness, $d$, (or size, $2r$, of the adsorbed objects) is known.\textsuperscript{15} If the interaction with the surface leads to nanoparticle deformation, the film thickness cannot, however, be directly deduced from the particle size in suspension, as we assumed in our previous work.\textsuperscript{20} An additional complication arises when determining the concentration of a specific sub-population of nanoparticles, e.g., CD63-positive EVs as exemplified in this work, since their mean size ($r^*$ in Figure 1) might differ from the mean size of the entire EV sample.

![Figure 1](image_url)

**Figure 1.** Schematic illustration of the key parameters that determine the magnitude and rate of a SPR response measured upon diffusion-limited binding of nanoparticles with a dimension, $r_{\text{mean}}$, that is on the order of the extension of the decay length, $\delta$. The left panel represents binding with equal probability of all nanoparticles, the rate of which is determined by $D$, which is inversely...
proportional to \( r_{\text{mean}} \). The magnitude of the measured response, \( R \), also depends on the fraction of the evanescent field that penetrates the nanoparticles, which is different depending on whether the attached particles are intact (\( d = 2r_{\text{mean}} \)) or deformed (\( d < 2r_{\text{mean}} \)) due to interaction with the surface. The right panel shows a somewhat more complex situation, when only a certain subpopulation (purple) of suspended nanoparticles with a mean radius, \( r^* \), different from \( r_{\text{mean}} \), binds to the surface, where they might also undergo surface-induced deformation.

Here, we address these challenges and revisit uncertainties and limitations of previous studies, with the aim to improve the precision by which SPR can be used to determine the bulk concentration of nanoparticles in general, and sub-populations of EVs, in particular. To reach this goal, we first introduce a generic SPR formalism for films, spherical nanoparticles (referred to as beads and experimentally represented by polystyrene beads), and spherical or deformed shells (experimentally represented by lipid vesicles) with dimensions approaching and exceeding \( \delta \). We then show how this formalism can be employed together with the dual-wavelength SPR mode, which enables simultaneous measurements with different \( \delta \) to offer a new means to determine both the adsorbed mass and the film thickness (or the size of adsorbed beads and vesicles). By combining the thickness determination using dual-wavelength SPR with size determination of suspended lipid vesicles using nanoparticle tracking analysis (NTA), it was possible to estimate the degree of surface-induced deformation, thereby resolving previously observed inconsistencies in concentration determinations.\(^{20}\) By also operating NTA in fluorescence mode, the size distribution of the EV sub-population carrying the biomarker CD63 (represented as \( r^* \) in Figure 1) was determined using fluorescently labeled antibodies directed against CD63 (anti-CD63). Combining information on the diffusion constant of EVs (as
determined from their size) and on their degree of deformation upon binding allowed us to markedly improve the accuracy of the determination of the bulk concentration of an EV sub-population. Thereby, the proposed approach emerges as clearly competitive to alternative quantification methods used for this purpose, including direct measurements of total protein mass,21 particle concentration determination using NTA,22 tunable elastomeric pore sensing method23 and quantifications of EV sub-populations using immunostaining combined with NTA operating in fluorescence mode24 or high-sensitivity variants of flow cytometry23.
EXPERIMENTAL SECTION

**Dual-wavelength surface plasmon resonance measurements**

The dual-wavelength surface plasmon resonance measurements were performed with a SPR Navi 220A (BioNavis, Finland). Polystyrene sulfate latex beads with nominal diameters of 80 and 140 nm (S37490 and C27366, respectively; Molecular Probes, Thermo Fisher Scientist, Waltham, MA, USA) were utilized for calibration of the system. The beads were bound to a gold-coated chip (SPR102-AU, BioNavis, Finland) treated with 50W oxygen plasma at 250 mTorr (Plasma Therm, BatchTop) for 30 sec. The chip was then incubated for 1 min. in 5%(w/v) aqueous solution of ACH (aluminium chlorohydrate, REHEIS Ireland) then rinsed in deionized water for 20 sec. and dried under nitrogen flow. The ACH coated chip was then docked in the SPR instrument and primed with circulating deionized water at 50 µL/min. Measurements were performed at 22°C and SPR was monitored at wavelengths 670 nm and 785 nm for a scanning interval between 65 and 78 degrees. Measurements on biotinylated liposomes (see Supporting Information) and EVs (see Supporting Information) were performed on gold-coated chips (SPR102-AU, BioNavis, Finland) first cleaned in a 5:1:1 solution of deionized water, 25% ammonia and 30% hydrogen peroxide for 10 minutes at 80 °C, then rinsed with deionized water, dried under a nitrogen flow, and incubated overnight in 50 mM oligo-ethylene-glycol disulfide ethanolic solution containing 99 mol% oligo-ethylene-glycol disulfides (dS-OEG) and 1 mol% oligo-ethylene-glycol disulfides with terminal biotin group (dS-OEG-biotin) (Polypure, Norway). After incubation, non-covalently bound disulfides were removed by ultra-sonicating the chip in ethanol for 2 minutes. The chip was then dried under nitrogen flow, docked in the SPR instrument and primed with circulating PBS buffer (Phosphate Buffer Saline, pH 7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate) at 10 µL/min. Sensitivity measurements were
acquired with xylitol (Sigma-Aldrich) dissolved at 300 mM in either PBS or TRIS-EDTA buffer [10 mM tris(hydroxymethyl)- aminomethane (Tris, Merck) and 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma)] and injected at 10 µL/min until a stable signal was obtained. Measurements on biotinylated liposomes were performed using the following injection sequence: 300 mM xylitol (PBS), NeutrAvidin at 25µg/mL in PBS at 10 µL/min until saturation of the signal, 300 mM xylitol (PBS), biotinylated liposomes at 100 µg/mL in PBS at 50 µL/min for 8 minutes, and finally 300 mM xylitol (PBS). Measurements on EVs were performed using the following injection sequence: 300 mM xylitol (PBS), NeutrAvidin at 25µg/mL in PBS at 10 µL/min until saturation of the signal, 300 mM xylitol (PBS), biotinylated anti-CD63 antibodies (353018, BioLegend, San Diego, CA) at 25 µg/mL in PBS at 10 µL/min until saturation, 300 mM xylitol (PBS), buffer exchange to TRIS-EDTA, 300 mM xylitol (TRIS-EDTA), EVs at 8.2 µg/mL total protein concentration (see Supporting Information) in TRIS-EDTA at 50 µL/min for 8 minutes or 10 µL/min for 40 minutes) and finally 300 mM xylitol (TRIS-EDTA). The bulk concentrations of EVs were determined using Eq. 18 with $\frac{\Delta \xi}{\Delta t}$ determined to ~0.13×10⁻³ deg/s obtained from a linear regression of the initial binding of EVs (Figure 7B), $\xi = 0.98(2/[h^2wl])^{1/3}$ where $h = 0.08$ mm, $w = 1.5$ mm, $l = 7.5$ mm, $S_{hi} = 121.5$ deg/RIU and $Q = 10$ µl/min.

**Nanoparticle tracking analysis**

The size distributions of biotinylated liposomes and unlabeled EVs were measured by particle tracking analysis, here referred to as nanoparticle tracking analysis, using a NanoSight LM10 module (Malvern Instruments Ltd, United Kingdom; 640 nm laser) in scattering mode. The camera and analysis settings were optimized to enable both size distribution and concentration determinations according to the manufacturer recommendations and were set as follows: camera
shutter: 1472, camera gain: 680, detection threshold: 6 and 4 for liposomes and EV suspensions, respectively, blur: 7×7, minimum track length: automatic, minimum expected particle size: 30 nm. All measurements were performed at room temperature and the buffer viscosity was assumed to be that of water. Each sample was measured 5 times for 1 minute under stagnant conditions with solution exchange between each measurement at a total lipid mass of 0.2 µg/mL for the two biotinylated liposome suspensions and at a total protein mass concentration of 1.1 µg/mL for the EV sample. The size distributions of EVs labeled with fluorescent anti-CD63 antibodies in scattering and fluorescence mode were measured by NTA using a NanoSight LM10 module (Malvern Instruments Ltd, United Kingdom; 488 nm laser). The measurements were performed at room temperature with temperature monitor control under flow conditions using a Nanosight syringe pump module. Fluorescence measurements were performed with adequate long path filter to only collect fluorescence emission. EVs stock solution (2µL) diluted in TRIS-EDTA (3µL) were incubated with PE-labeled anti-CD63 (clone H5C6, BD Biosciences; 5µL of stock solution) for 30 min. at 4 °C at a final EV protein mass concentration of 0.26 mg/mL. The suspension was then filtered to remove unbound anti-CD63 antibodies using size exclusion columns (MicroSpin S-200 HR, GE Healthcare). Prior to filtration, the buffer present in the column was exchanged with TRIS-EDTA buffer according to manufacturer recommendations by spinning 3 times 300 µL of PBS through the column at 740 x g for 2 min. The filtration step did not influence the concentration or size distribution according to scattering based NTA data (not shown). The EVs suspension incubated with antibodies was then diluted 3 times and the final 30 µL suspension was filtered through a size exclusion column at 740 x g for 2 min. The filtered solution of EVs labeled with anti-CD63 antibodies was then diluted in TRIS-EDTA to final volume of 1 mL and measured with NTA both in scattering and fluorescence mode 3 times for
90 sec. Camera and analysis settings used for scattering (S) and fluorescence (F) were: camera shutter: 30.15 ms (S) and 32.5 ms (F), camera lower and upper histogram: 95 and 3294 (S) and 95 and 650 (F), detection threshold: 3 (S) and 8 (F).

PHYSICAL BACKGROUND OF THE SPR RESPONSE

The SPR response, $R$, is defined as the shift in either wavelength or angle of the SPR minimum in reflected light intensity associated with changes in the index of refraction of the medium in contact with the metal surface of the SPR device. For a film of thickness $d_f$, it can be represented as $^{15}$

$$ R = S(n_f - n_b)[1 - \exp(-d_f/\delta)] $$

where $n_b$ and $n_f$ are the bulk and film refractive indices, $\delta$ is the decay length of the intensity (square of the amplitude) of the exponentially vanishing SPR evanescent field, and $S$ is the sensitivity factor, usually expressed in SPR signal per change in bulk refractive index unit. For our analysis, it is convenient to rewrite Eq. (1) as

$$ R = S(n_f - n_b)d_f\delta^{-1}\varphi(d_f/\delta) $$

where

$$ \varphi(d_f/\delta) = [1 - \exp(-d_f/\delta)]\delta/d_f $$

is a dimensionless factor taking into account that $d_f$ may be comparable or larger than $\delta$ (for thin films with $d_f \ll \delta$, this factor is close to unity). In addition, we take into account that the concentration (per unit volume), $c_*$, of molecules forming a film can be represented as (for the derivation, one can divide the left and right sides of Eq. (10) in Reference $^{26}$ by $d_f$):

$$ c_* = \Delta \Gamma_f / d_f = \frac{n_f - n_b}{dn/dC_*} $$

(4)
where \( dn/dC \) is the derivative of the refractive index with respect to the molecule concentration in solution. Using Eq. (4), one can express \((n - n_b)\) via \( c_* \) and then rewrite Eq. (2) as

\[
R = S(dn / dC_*)c_*d_i \delta^{-1} \varphi(d_i / \delta) \tag{5}
\]

In this expression, \( c_*d_i = \Delta \Gamma \) represents the number of molecules in a film per unit surface area, \textit{i.e.}, the surface coverage as conventionally referred to in the SPR literature.

The prescriptions used in Reference 15 to derive Eq. (1) for a film (and similar equations for cylindrically shaped islands) can be employed to generalize Eqs. (3) and (5) to the case of a supported layer of nanoparticles of arbitrary shape. In particular, the expression for the SPR signal can be represented as

\[
R = S(dn / dC_*)c_*c_c \nu_m \delta^{-1} \varphi \tag{6}
\]

where \( \nu_m \) is the integral volume of molecules forming a nanoparticle (if a nanoparticle has no hollows, this is its volume), \( c_* \) is the concentration (per unit volume) of molecules in nanoparticles, \( c_\text{c} \) is the concentration of nanoparticles, and \( c_*c_c \nu_m \) is the number of molecules in a layer per unit surface area [this number plays the same role as \( c_*d_i \) in Eq. (5)]. To obtain the expression for \( \varphi \), we take into account that the contribution of a single nanoparticle to the SPR signal is proportional to the following integral (cf. Eq. (3) in Reference 15)

\[
I = \int_{0}^{\nu_m} \frac{d \nu(z)}{dz} \exp(-z / \delta) dz \tag{7}
\]

where \( z \) is the coordinate perpendicular to the surface \((z = 0 \text{ corresponds to the surface})\), \( \nu_m \) is the maximum value of \( z \) in a nanoparticle, and \( \nu(z) \) is the integral volume of molecules in a nanoparticle in the region from 0 to \( z \) (according to this definition, we have \( \nu(z_*) = \nu_m \)). If \( z_* << \)
\(\delta\), one can replace \(\exp(-z/\delta)\) by unity, and \(I\) will be equal to \(v_m\). Thus, \(\varphi\) is given by the ratio of \(I\) and \(v_m\), i.e.,

\[
\varphi = \frac{I}{v_m} = \int_0^\infty \frac{dv(z)}{dz} \exp(-z/\delta) dz / v_m
\]

For a layer of spherical beads attached to the surface, Eq. (6) yields

\[
R = S(dn / dC_\ast)cc_s(4\pi r^2 / 3)\delta^{-1} \varphi(r/\delta)
\]

where \(r\) is the bead radius. Taking also into account that in this case \(dv(z)/dz = \pi[r^2 - (r - z)^2]\) and \(z_\ast = 2r\) and substituting these relations into Eq. (8) result in

\[
\varphi(r/\delta) = 0.75[F_1(r/\delta) - F_2(r/\delta)]
\]

where

\[
F_1(x) = x^{-1}[1 - \exp(-2x)] \quad \text{and}
\]

\[
F_2(x) = x^{-3}[x^2 - 2x + 2 - (x^2 + 2x + 2)\exp(-2x)]
\]

For a layer of vesicles attached to the surface, Eq. (6) instead yields:

\[
R = S(dn / dC_\ast)cc_v(4\pi r^2 l)\delta^{-1} \varphi,
\]

where \(dn / dC_\ast\) is the derivative for the reference case (i.e., with respect to the lipid concentration in solution), \(r\) is the vesicle radius in the undeformed state, and \(l\), \(4\pi r^2\) and \(4\pi r^2 l\) are the bilayer thickness, area and volume respectively (these expressions are applicable irrespective whether a vesicle is deformed or not). If vesicles are undeformed, Eq. (8) results in

\[
\varphi(r/\delta) = [1 - \exp(-2r/\delta)]\delta / 2r
\]

By comparing expressions (3) and (14) for the size-dependent factor \(\varphi\) one can first of all conclude that the results for a film and undeformed vesicles are mathematically identical [note that \(2r\) in (14) can be replaced by the vesicle diameter, \(d = 2r\), which plays the same role as the
film thickness, \( d_i \), in Eq. (3)]. This means that \( d_i \) and \( d \) can be used interchangeably (for this reason, we omit in the following the subscript \( f \) in \( d_f \)).

As detailed in the Supporting Information and shown in Figure S1, \( \varphi \) is nearly identical for beads and undeformed vesicles provided that \( r/\delta < 1 \). Since \( \delta \) is typically larger than 100 nm, this matches the region that is of practical interest in this work.

RESULTS AND DISCUSSION

**Determination of film thickness from dual-wavelength SPR response**

The possibility to use dual-wavelength SPR to determine the film thickness (or size) of adsorbed nanoparticles stems from the fact that several of the physical parameters of homogeneous films, spherical beads as well as undeformed and deformed hollow vesicles that determine the SPR sensor response [cf. Eqs. (6), (9) or (13)] depend on the illumination wavelength, \( \lambda \). These include the sensitivity factor, \( S \), usually expressed in SPR signal per change in bulk refractive index unit, the derivative of the refractive index with respect to the molecule concentration in solution, \( dn/dC_*, \) the decay length of the intensity (square of the amplitude) of the exponentially vanishing SPR evanescent field, \( \delta \), and the dimensionless factor \( \varphi \) [see Eq. (14)], which takes into account that the film thickness (or the object size) may be comparable or larger than \( \delta \). Consequently, if the SPR response, \( R \), is measured at two different wavelengths (\( \lambda_1 \) and \( \lambda_2 \)), we have from Eq. (13) that

\[
\frac{R_{\lambda_1}}{R_{\lambda_2}} = \frac{S_{\lambda_1} (dn/dC_*)_{\lambda_1} \varphi_{\lambda_1} \delta_{\lambda_1}}{S_{\lambda_2} (dn/dC_*)_{\lambda_2} \varphi_{\lambda_2} \delta_{\lambda_2}}
\]  

(15)
Importantly, if $S$, $dn/dC_*$ and $\delta$ are known, the specific dependences [e.g. Eqs. (10), (14) and (S1)] of $\varphi$ on $d$ (or $2r$, where $r$ is the nanoparticle radius) make it possible to directly determine $d$ from the measured $R_\lambda / R_{\lambda_2}$ ratio. This is illustrated in Figure 2 and discussed together with experimental results (also summarized in Figure 2) in further detail below, with emphasis on calibration using bulk refractive index measurements, a protein film with $d << \delta$, validation of the thickness determination by employing monodisperse rigid spheres, and application to adsorbed lipid vesicles in general and EVs in particular.

**Figure 2.** The $R_{\lambda_1} / R_{\lambda_2}$ ratio versus $d$ or $2r$ for $\lambda_1 = 670$ nm and $\lambda_2 = 785$ nm plotted. The red line corresponds to Eq. (15) with $(dn / dC_*)_{\lambda_1} \approx 1.02 (dn / dC_*)_{\lambda_2}$, $S_{\lambda_1} / S_{\lambda_2} = 1.185$ (determined from calibration measurements), and $\delta_{\lambda_1} = 109$ nm and $\delta_{\lambda_2} = 154$ nm. The blue lines take an uncertainty of +/- 5% in the determination of $\delta_{\lambda_1}$ and $\delta_{\lambda_2}$ into account, with the ratio kept fix at 1.415 (see main text). The vertical columns represent representative experimental data (with the coloured bars indicating the statistical error for a few measurements) for two sets of polystyrene beads with nominal diameters of 80 and 140 nm, respectively (orange
area; see Fig. 4), synthetic lipid vesicles (green area; see Fig. 5) and two batches of CD63-positive EVs (blue area; see Fig. 7). Note that $\varphi$ was determined using Eq. (14) rather than Eq. (10), since when $r/\delta \leq 2$, Eq. (14) is a good approximation, as illustrated in Figure S1.

**Calibration of the dual-wavelength SPR system**

The bulk sensitivity parameters $S_\lambda$ and $S_\varphi$ for the dual-wavelength SPR system used in this work were previously determined to be 121.5 and 102.8 deg/RIU, respectively,\textsuperscript{28} which yields $S_\lambda / S_\varphi = 1.182$. This is in excellent agreement with the ratio of 1.185 measured upon addition of xylitol (Figure 3) to a sensor surface modified with a thin (~2 nm) biotin-thiol film (see Experimental Section). The response upon subsequent addition of the protein NeutrAvidin, forming a ~5 nm thin film\textsuperscript{29}, resulted in $R_{\lambda_1} / R_{\lambda_2} = 1.71$ (Figure 3). In this thin-film regime ($d << \delta$), $\varphi$ becomes approximately 1, and with $(dn / dC_x)_{\lambda_1} \approx 1.02(dn / dC_x)_{\lambda_2}$ for proteins\textsuperscript{27}, Eq. (15) simplifies to

$$\frac{R_{\lambda_1}}{R_{\lambda_2}} = 1.02 \frac{S_{\lambda_1} \delta_{\lambda_2}}{S_{\lambda_2} \delta_{\lambda_1}}$$  

(16)

With $R_{\lambda_1} / R_{\lambda_2} = 1.71$ and $S_{\lambda_1} / S_{\lambda_2} = 1.185$, the $\delta_{\lambda_2} / \delta_{\lambda_1}$ ratio becomes 1.415, which is in excellent agreement with theoretical simulations in a water environment ($n_b = 1.33$), yielding $\delta_{\lambda_2} = 154$ and $\delta_{\lambda_1} = 109$ nm, and a $\delta_{\lambda_2} / \delta_{\lambda_1}$ ratio of 1.413.\textsuperscript{28} Since the absolute values of the decay lengths were theoretically estimated, and therefore subject to some uncertainties (e.g., due to the choice of dielectric constant of thin gold films\textsuperscript{30}), the analysis was performed for decay lengths with absolute values that varied within an interval of $\pm$5%, but with the ratio $\delta_{\lambda_1} / \delta_{\lambda_2}$ kept fixed at the experimentally determined value of 1.415 (Figure 2, blue lines).
**Figure 3.** Dual-wavelength SPR sensorgrams showing the angle shift, $R$, versus time (red: $\lambda_1 = 670$ nm; blue: $\lambda_2 = 785$ nm) upon subsequent additions of i) 300 mM xylitol and ii) 25 $\mu$g/mL NeutrAvidin to a biotin-modified sensor chip. Also shown is the corresponding $R_{\lambda_1}/R_{\lambda_2}$ ratio versus time (open grey diamonds) for steps i) and ii).

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**Determination of the size of adsorbed beads and lipid vesicles**

With this calibration at hand, Figure 2 offers a direct means to characterize the geometry of adsorbed nanoparticles. This was verified by adsorbing homogenous polystyrene beads with nominal diameters of 80 nm and 140 nm on the sensor surface (see Experimental Section), yielding ratios of 1.47 and 1.55, respectively (Figure 4).
Figure 4. As Figure 3, but for adsorption of polystyrene beads (nominal diameter 140 nm) to an aluminium chlorohydrate coated sensor surface, excluding the xylitol addition step.

From the intercept of the measured $R_{\lambda_1} / R_{\lambda_2}$ ratios with the curve representing Eq. (15) (Figure 2, red line), and with the uncertainty imposed by the +/- 5% variation in the absolute values of the decay lengths taken into account, the sizes of the small and large polystyrene beads were determined to be 82 +/- 4 nm and 137 +/- 10 nm, respectively. This is in good agreement with AFM measurements indicating mean diameters of 80 nm and 137 nm (see Supporting Information), verifying that in these cases the particle size can be determined with an uncertainty of less than +/- 5%. This uncertainty can be slightly improved by determining $\varphi$ using Eq. (10) instead of Eq. (14).

Note that in Figure 4, $R_{\lambda_1} / R_{\lambda_2}$ is subject to somewhat higher noise at low surface coverage. If this ratio was instead determined from a linear fit to the initial response, essentially the same value of particle size was obtained, indicating small dependence of the surface coverage on $R_{\lambda_1} / R_{\lambda_2}$. As detailed in the Supporting Information, this is in agreement with theoretical
predictions using the Fresnel formalism, showing that the measured \( \frac{R_{\lambda_1}}{R_{\lambda_2}} \) ratio is expected to vary by less than 1% with coverage or film thickness in the refractive index interval of relevance for adsorbed beads, vesicles and EVs (see Figure S3).

While the determined size of polystyrene beads is in good agreement with their independently measured mean diameter, a pronounced difference between the diameter in bulk and \( d \) was measured for synthetic biotinylated POPC vesicles adsorbed on a NeutrAvidin-functionalized surface (see Experimental Section). In particular for vesicles with a nominal diameter of 170 nm (Figure 5A), the measured \( \frac{R_{\lambda_1}}{R_{\lambda_2}} \) ratio of 1.52 +/- 0.02 (3 experimental repeats; see e.g. Figure 5B) translates into a film thickness of 105 +/- 5 nm (Figure 2). This suggests a significant adsorption-induced vesicle deformation, an interpretation that is further supported by recent AFM and LSPR studies of similar systems. During the adsorption process, there was also a slight decrease of \( \frac{R_{\lambda_1}}{R_{\lambda_2}} \), suggesting a slight increase in the effective film thickness with increasing vesicle coverage.

**Figure 5.** A) Size distribution of synthetic lipid vesicles without (red) and with (green) 30 wt% cholesterol as determined using NTA. B) As Figure 4, but for adsorption of biotin-containing lipid vesicles to a sensor substrate modified with NeutrAvidin (see Experimental Section).
Influence of vesicle deformation on bulk-concentration determination of suspended vesicles

We now turn to the possibility to improve the accuracy by which the rate of mass deposition can be translated into the bulk concentration of suspended nanoparticles aided by knowledge about surface-induced vesicle deformation. If the supply of nanoparticles to a surface is globally controlled by diffusion in solution, their concentration on the surface, $c(t)$, can be accurately calculated by solving Fick’s equation with the adsorbing boundary condition. Under the steady-state flow conditions one has\textsuperscript{20}

$$c(t) = \xi(D^2Q)^{1/3}t$$

(17)

where $D$ and $C$ are the nanoparticle diffusion constant and bulk concentration, respectively, $Q$ the volumetric flow rate, $\xi$ a constant related to the geometry of the flow cell (see Experimental Section), and $t$ is the time defined such that $t = 0$ corresponds to the onset of adsorption. Combining Eqs. (6), (9) or (13) and (17) makes it possible to determine the nanoparticle concentration in solution. For example, substituting (17) into (6) yields

$$C = R(t) / [A \xi(D^2Q)^{1/3}t]$$

(18)

where $A = S(dn/dC)\varepsilon\nu_n \delta^{-1}\varphi$.

Note that Eq. 18 depends both on the film thickness (or size of adsorbed nanoparticles), via the dependence of $R$ on $d$ or $2r$ [see e.g. Eq. (13) and (14)], and on the diffusion constant, which is inversely proportional to the size of suspended nanoparticles. Since adsorption measurements monitored with dual-wavelength SPR under diffusion-limited conditions permit us to simultaneously determine both the size of adsorbed nanoparticles (Figure 2) and the rate by which they bind to the surface (Figure 5B), such combined information opens up a novel
possibility to improve the bulk concentration determination, which can be extracted from the SPR temporal response $R(t)$ in combination with Eq. (18)

In earlier use of Eq. (18) to determine the bulk concentration of synthetic lipid vesicles, their surface-induced deformation was assumed to be negligible, which resulted in an overestimation of their bulk concentration by 50% compared to that determined by employing the total lipid mass used to prepare the vesicles.$^{20}$ One likely explanation to that discrepancy was suggested to be adsorption-induced vesicle deformation, since this effect is expected to cause an increase in the SPR response, which directly translates into an error in the concentration determination. To clarify this aspect of the use of Eq. (18), we employ here Eqs. (13), (14) and (S1) and describe the difference in SPR response of undeformed vesicles:

$$R_{\text{circ}} \propto 4\pi r^2 \left[1 - \exp \left(-\frac{2r}{\delta}\right)\right]/2r$$

and deformed vesicles (represented as truncated spheres):

$$R_{\text{def}} \propto \frac{\pi a^2}{\delta} + 2\pi \rho \left(1 - \exp\left\{-\rho + \left(\rho^2 - a^2\right)^{1/2}\right\}/\delta\right)$$

where $r$, $a$ and $\rho$ are defined as shown in Figure 6A. Recalling our previous results for vesicles with a nominal diameter of 170 nm probed with an SPR decay length of 150 nm,$^{20}$ a deformation leading to a decrease of vesicle height from 170 nm (Figure 5A) down to 100 nm (Figure 2, green area) yields, according to Eqs. (19) and (20), an overestimation of the bulk concentration [as determined from Eq. (18)] by $\sim 37\%$ (Figure 6B, red dots).
Figure 6. A) Definition of the parameters used to represent vesicle deformation upon adsorption onto a solid substrate: (a) no deformation, (b) weak deformation and (c) strong deformation. B) Influence of the vesicle deformation (expressed by the ratio of vesicle height after adsorption, $d$, and vesicle diameter in suspension, $2r$) on the bulk concentration as determined using Eq. (18) (given in percentage of overestimation with respect to the true value) for $\delta = 150$ nm and $2r$ ranging between 50 and 400 nm as indicated in the plot.

This shows that the previous discrepancy observed using SPR to determine the bulk concentration of lipid vesicles (having a diameter in the range of the decay length) can be almost fully accounted for by vesicle deformation. Consequently, by directly measuring the degree of deformation using SPR as done here, one can determine the bulk concentration with an error of 10% or less, when uncertainties with respect to the absolute value of $dn/dC$ for lipid vesicles and a deviation in size distribution in bulk and on the surface are taken into account.

Determination of the bulk concentration of a sub-population of EVs
With the possibility of using NTA and dual-wavelength SPR to determine both the nanoparticle size, their surface-induced deformation and thus bulk concentration, we finally turn our attention to the significantly more heterogeneous and analytically challenging sample of extracellular vesicles. Briefly, a suspension of EVs isolated from conditioned media of the human mast cell line, HMC-1.2 (see Supporting Information), was analyzed by probing the rate of binding under diffusion-limited conditions, as detailed in Reference 20, to a surface modified with IgG antibodies directed against CD63 (see Experimental Section and Figure S5), a tetraspanin membrane protein marker known to be present on EV samples of this type\(^{36}\) and often considered a marker for exosomes\(^{37}\).

The size distribution of this sub-population in the suspended state was determined by employing NTA in the fluorescence mode using fluorescently labeled anti-CD63 antibodies (Figure 7A). This analysis showed that the fraction of CD63-positive EVs had a relatively narrow size distribution with a mean diameter of ~80 nm (Figure 7A, inset), which was markedly smaller than the mean diameter of ~170 nm of the entire EV sample, and a concentration corresponding to less than ~5% of the entire EV sample (Figure 7A). The latter observation was supported by total protein concentration measurements of CD63-positive EVs obtained after four extraction cycles using anti-CD63 antibody functionalized beads (not shown), indicating a remaining protein content of 6.5%.
Figure 7. A) Diameter distributions of an EV sample fluorescently labelled with anti-CD63 antibodies as determined using NTA operated in scattering (blue) and fluorescence (red) mode (inset: same data but normalized to the peak value). B) As Figure 5B, but for adsorption of EVs to a sensor substrate modified with anti-CD63 antibodies. All injections steps of this measurement are shown in Figure S6 in Supporting Information.

The measured rate of EV binding (Figure 7B) and a mean diameter of ~50 nm [estimated from the size of adsorbed EVs (Figure 2)], yields according to Eq. (18) a bulk concentration of CD63-positive EVs of ~1.5 μg/ml, which should be compared with the total protein content of ~8 μg/ml determined for the EV solution used in this experiment (see Supporting Information). Taking into consideration that at least 50% of the mass of EVs is expected to originate from lipids, nucleotides, glycans etc, this suggests that ~10% of all EVs are CD63-positive. By instead assuming a mean diameter of suspended EVs of 80 nm (as estimated using fluorescence NTA, Figure 7A) and an adsorption-induced deformation of 60% (as estimated using dual-wavelength SPR, Figure 2), the overestimation of the bulk concentration becomes no more than
~20% (Figure 6B) with a value of 1.6 μg/ml – a number that is still very close to the 1.5 μg/ml obtained by assuming a diameter of ~50 nm and no deformation.

A concentration of 1.5 to 1.6 μg/ml corresponds to around 10% of the total EV concentration, which is larger than the 5% obtained by comparing the fluorescence and scattering-based NTA histograms (Figure 7A). However, in the case of NTA it cannot be excluded that there is a significant number of EVs that either contains too few CD63 markers to be detectable or carries antibodies a decorated with a low number of dye molecules. For example, titration curves performed with NTA in fluorescence mode using lipid vesicles with decreasing number of fluorescently labelled lipids indicated that a drop in the number count was observed below ~10 to 20 dyes per lipid vesicles (not shown). With a maximum of 5 to 10 dyes per antibody, this indicates that at least five CD63 need to be present per EV to be clearly detectable in NTA, provided that there is a 1:1 relation between CD63 and bound antibodies. Considering also that the number of CD63 decreases with reduced EV size, one expects a preferential reduction in detection efficiency for small compared with large EVs, i.e., the mean diameter of CD63-positive EVs measured with NTA is expected to be shifted towards larger rather than smaller diameters. This is in agreement with the size obtained from an electron microscopy (EM) analysis summarized in the Supporting Information (Figure S6), although EM bases the size determination from the electron density of the uranyl acetate used for staining rather than the hydrodynamic radius obtained using NTA. Further, already a single CD63 per EV should be sufficient for efficient binding to the anti-CD63 modified surface, which also yielded a size of around 50 nm for the adsorbed EVs (Figure 2). Consequently, the accuracy of the concentration determination EV sub-populations using SPR is likely to be better than that of NTA, although
one must recall that there is a significant uncertainty connected with the conversion of mass per volume to number of particles per volume.

It was also observed that upon binding of CD63-positive EVs, there was a time-resolved decrease of $R_{\Delta_1}/R_{\Delta_2}$ (from 1.62 to 1.57) upon increased coverage (Figure 7B). This observation contradicts the theoretical expectation (increase in $R_{\Delta_1}/R_{\Delta_2}$ with surface coverage; Figure S3), suggesting that this feature may have another origin. In particular, a time-dependent deformation of adsorbed EVs would correspond to a temporal increase of $R_{\Delta_1}/R_{\Delta_2}$, rather than the experimentally observed decrease. Hence, a plausible explanation to this feature is that the initial adsorption is diffusion-limited, while binding of larger entities [the presence of which is supported by the NTA analysis (Figure 6A, inset)] might dominate at later stages, at which the binding could also be kinetically controlled. The latter conclusion remains, however, speculative, and has no significant impact on the major conclusions regarding the accuracy by which the bulk concentration of EV subpopulations can be determined.

CONCLUSIONS

Previously, dual-wavelength SPR measurements were primarily employed to characterize homogeneous films$^{30,38-39}$ and to our knowledge for adsorbed beads only once$^{40}$, although in the latter case not treated in detail. The improved formalism presented in this work encompasses the SPR response of homogeneous films, adsorbed beads and both deformed and undeformed lipid vesicles. Combined with dual-wavelength SPR measurements the approach was shown to enable determination of the film thickness or size of adsorbed nanoparticles in a convenient manner and with relatively high accuracy.

Further, aided by operating SPR in dual-wavelength mode under diffusion-limited binding conditions, information about adsorption-induced deformation was used to improve the
determination of the bulk concentration of biological nanoparticles – here demonstrated for synthetic lipid vesicles – and to determine the concentration of a sub-population of nanoparticles upon specific binding to a functionalized sensor surface – here demonstrated for CD63-positive EVs using surface immobilized anti-CD63 antibodies. It should be noted, though, that the formalism presented above corresponds to adsorption of nanoparticles (beads or vesicles) of one type. If the solution contains nanoparticles having broad size distributions, the integral SPR response, $R$, can be represented as a sum of responses of different nanoparticles. This complication was treated previously, and corresponds to a maximum error of 10% for the systems investigated in this work.

Surface-based capturing of different EV sub-populations also opens up the possibility of identifying patterns of multiple biomarkers present on different EVs. Until label free-single vesicle approaches, such as interferometric or waveguide scattering microscopy techniques, are sensitive enough to unambiguously detect each EV that binds to a surface, ensemble averaging methods like SPR have their own merits, since they allow every interaction to count. We therefore await how this field will develop with excitement and how the contribution presented in this work will position itself as a metrology tool for quantitative analysis of biological nanoparticles in general and of EVs and exosomes in particular.

ACKNOWLEDGEMENTS

The authors would like to thank Agnieszka Siupa for her support and expertise with NTA measurements. This project was funded by the Swedish Research Council (VR), the Swedish Governmental Agency for Innovations Systems (VINNOVA) and the Göran Gustafsson foundation.
ASSOCIATED CONTENT

**Supporting Information.** Contains Further comments on the Physical background of the SPR analysis, Surface functionalization for capturing of CD63-positive extracellular vesicles, Electron microscopy inspection of EVs and Complementary experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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

**Notes**

The authors declare no competing financial interest.
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