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Published in: Nature Communications

Link to article, DOI: 10.1038/s41467-020-15889-3

Publication date: 2020

Document Version Publisher's PDF, also known as Version of record

Citation (APA): Rasmussen, M. K., Pedersen, J. N., & Marie, R. (2020). Size and surface charge characterization of nanoparticles with a salt gradient. Nature Communications, 11(1), [2337]. https://doi.org/10.1038/s41467-020-15889-3

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Size and surface charge characterization of nanoparticles with a salt gradient

Exosomes are nanometer-sized lipid vesicles present in liquid biopsies and used as biomarkers for several diseases including cancer, Alzheimer's, and central nervous system diseases. Purification and subsequent size and surface characterization are essential to exosome-based diagnostics. Sample purification is, however, time consuming and potentially damaging, and no current method gives the size and zeta potential from a single measurement. Here, we concentrate exosomes from a dilute solution and measure their size and zeta potential in a one-step measurement with a salt gradient in a capillary channel. The salt gradient causes oppositely directed particle and fluid transport that trap particles. Within minutes, the particle concentration increases more than two orders of magnitude. A fit to the spatial distribution of a single or an ensemble of exosomes returns both their size and surface charge. Our method is applicable for other types of nanoparticles. The capillary is fabricated in a low-cost polymer device.
 purification and analysis of nanoparticles, as routinely performed when isolating exosomes from liquid biopsies, are often based on size and surface properties, for example, their biochemical components or zeta potential. The same parameters are also important for the synthesis and functionality of both solid state and soft matter synthetic colloids. These can, for example, be designed to promote interactions with living cells, have active reaction sites on their surfaces, or show specific optical properties.

The zeta potential, which depends on the surface charge, is important for the stability of nanoparticles in suspension and is also the major factor in the initial adsorption of nanoparticles onto the cell membrane. After adsorption, the endocytotic uptake rate depends on the particle size. The zeta potential and size thus affect nanoparticle toxicity.

Exosomes, present in most body fluids, have received great interest due to their potential as biomarkers and use in precision medicine. The zeta potentials and sizes of exosomes from various body fluids are significantly different as they interact with different cellular targets. So these parameters reflect both the origin and the endocytotic pathway that the exosomes may use, and they are integral properties of the cell-to-cell signaling system. Control of the size and zeta potential are thus important factors for the effectiveness of nanoparticles for drug delivery, and allow for specifying the cellular targets for, for example, liposomes, gold nanoparticles, and copolymer micelles. To combine the advantages of size-based concentration methods, is time consuming and suffers from contamination. Size-based filtration through membranes can result in deformations or even break the particles. Current characterization techniques for nanoparticles also all have challenges, for example, cryo-electron microscopy that is a destructive method. Measurements of size and zeta potential are routinely performed on the same instrument by dynamic light scattering (DLS) and laser Doppler electrophoresis (LDE), but require multiple experiments, and are challenged by polydisperse samples, for example, exosomes isolated from body fluids.

The fast development within micro- and nanofluidics allows for reduced reagent consumption in lab-on-a-chip devices. Microfluidics-based purification and characterization methods have become increasingly important for nanoparticle research in general, and in particular for soft matter nanoparticles. In micro- and nanofluidic devices, nanoparticles can be exposed to gradients in, for example, electric potential, temperature, and solute concentration, and such gradients induce phoretic transport processes that depend on particle properties.

In an electric field, particles experience electrophoretic transport, which is, for example, utilized in size-dependent separation of DNA molecules in microfluidic devices. Different salt concentrations in the two microchannels maintain a position-dependent salt concentration $C(x)$ across each nanochannel. The salt concentrations are identical in all nanochannels, that is, there are 16 parallel experiments on the device. The salt gradients in the nanochannels cause a diffusiophoretic particle migration and an oppositely directed diffusiomotic fluid flow, and there are multiple experiments on the device. The boundary conditions of the nanochannel is $C_N$ at the narrow and wide ends of the nanochannel, respectively.

Diffusion determines the salt gradient in each funnel-shaped nanochannel. The boundary conditions are the fixed salt concentrations in the microchannels, that is, $C_N$ at the narrow and wide ends of the nanochannel, respectively. The concentration profile of the trapped nanoparticles $C_p(x)$ depends on their size and zeta potential.

Diffusion is defined as $D = \frac{\partial^2}{\partial x^2}$, where $x$ is the local width of the nanochannel. We parameterize it as $w(x) = w_N + \Delta w x / L$, with $\Delta w = w_w - w_N$, where $w_w = 20 \mu m$ and $w_N = 5 \mu m$ are the widths of the nanochannel at the wide and narrow ends of the nanochannel, respectively. The length of the nanochannel is $L = 440 \mu m$.

The salt gradient results in a diffusiophoretic particle velocity

$$v_{ph}(x) = \Gamma_{ph}(d, \zeta) \partial_x \ln C(x).$$

Here $\Gamma_{ph}$ is the diffusiophoretic mobility, which depends on the particle diameter $d$ and zeta potential $\zeta$ (for details, see “Methods”).
The diffusioosmotic fluid flow in the nanochannel is due to the local diffusioosmotic slip velocity $v_{\text{slip}}(x)$. For a fixed solute gradient in bulk near a charged wall, the diffusioosmotic slip velocity is

$$v_{\text{slip}}(x) = -\Gamma_{\text{os}}(\zeta_{\text{ch}}) \partial_x \ln C(x),$$  \hspace{1cm} (3)$$

where $\Gamma_{\text{os}}(\zeta_{\text{ch}})$ is the diffusioosmotic mobility and $\zeta_{\text{ch}}$ is the zeta potential of the channel wall (details in “Methods”). The diffusioosmotic slip velocity causes a constant fluid flow rate $Q$ due to conservation of mass, but the fluid velocity varies because of the changing channel width. We introduce a position-dependent diffusioosmotic flow velocity

$$v_{\text{os}}(x) = \frac{Q}{hw(x)},$$  \hspace{1cm} (4)$$

where $h = 240$ nm is the height of the nanochannel.

We then relate the diffusioosmotic flow velocity in the nanochannel $v_{\text{os}}(x)$ to the diffusioosmotic slip velocity $v_{\text{slip}}(x)$ by assuming that

$$Q = w(x)h v_{\text{slip}}(x) - \frac{w(x)h^2 \partial_x P(x)}{12\eta},$$  \hspace{1cm} (5)$$
Fig. 2 Tracking a single, trapped particle. a Fluorescence microscopy image of a single, trapped liposome. Salt concentrations in the microchannels are fixed at $C_{D}$ equal to $10^{-3}$ X PBS and $C_{W}$ equal to $10^{3}$ PBS, that is, $\ln(C_{D}/C_{W}) = -9.2$. Scale bar is 10 μm. b Measured positions in the nanochannel for the area indicated with the red box in a. The particle is tracked for $T_{\text{max}} = 40$ s and imaged at 20 frames per second. c x-Coordinates versus time. d Histograms and fitted distributions obtained from single-particle tracking of liposomes from three different populations with different diameters and zeta potentials. Upper panel corresponds to data in c. e Diameters and zeta potentials of individual particles from the POPC:POPG 3:1 population with $d_{\text{ave}} = (160 \pm 16)$ nm and $\zeta_{\text{LDE}} = (-28 \pm 1)$ mV. Results for particle no. 1 are from the upper panel of d. Error bars are the standard deviations obtained from the fits to histograms in d.

holds locally, as the width is slowly varying compared to the length of the nanochannel ($\Delta w/L \ll 1$). Here $\partial_{x} P(x)$ is the internal pressure gradient in the fluid along the axis of the channel. It is assumed that the pressures are identical at the two ends of the nanochannel, $P(0) = P(L)$. Dividing both sides in Eq. (5) with $w(x)$ and integrating from 0 to $L$ using the boundary conditions on the pressure gives $Q = \tau_{\text{w}}(C_{D}) \ln(C_{D}/C_{W}) \Delta w/[L \ln(1 + \Delta w/w_{N})]$, and, consequently, $v_{\text{os}}(x)$, see Eq. (4). Note that the flow rate only depends on the salt concentrations in the microchannels, not the specific form of the salt concentration in the nanochannel.

Particles are trapped at the position $x_{0}$, where $v_{\text{os}}(x_{0}) + v_{\text{ph}}(x_{0}) = 0$. In a straight channel, the diffusiophoretic flow velocity $v_{\text{os}}(x)$ is constant along the channel, but our funnel-shaped nanochannel gives a varying fluid velocity. This results in a tighter trap.

Finally, we calculate the particle concentration in the nanochannel $C_{p}(x)$. It is related to the particle current density along the x-axis $J_{x}(x)$ (as Fick’s first law of diffusion with drift)

$$J_{x}(x) = -D_{p} \partial_{x} C_{p}(x) + \left[ v_{\text{ph}}(x) + v_{\text{os}}(x) \right] C_{p}(x). \quad (6)$$

Here $D_{p} = D_{p}(d)$ is the size-dependent diffusion coefficient of particles in the nanochannel, which differs from its bulk value due to the interactions with the walls of the nanochannel (see “Methods”). The particle current $I_{x}(x)$ in the nanochannel equals the particle current density times the cross-sectional area of the nanochannel, that is, $I_{x}(x) = hw(x)J_{x}(x)$. In steady state, $I_{x}(x)$ is constant along the nanochannel, and if particles are trapped, it vanishes. So $I_{x}(x) = 0$, and, consequently, $J_{x}(x) = 0$. For a trapping position $x_{0}$ far away from both ends of the nanochannel, a solution to the particle concentration is (see Eq. (6))

$$C_{p}(x) = \frac{N}{[hw(x_{0})L_{\text{trap}}]}, \quad \text{where } N \text{ is the number of trapped particles and } L_{\text{trap}} = \int_{x_{0}}^{x_{\text{trap}}} \frac{dx}{[w(x)/w(x_{0})]\exp\left[\int_{x_{0}}^{x} \frac{dx'}{[v_{\text{ph}}(x')/D_{p}]}\right]} \text{ is the effective length of the trap. That is, the particle distribution is normalized such that an integration over the volume of the nanochannel gives the number of trapped particles, } \int_{-\infty}^{x_{\text{trap}}} \int_{-\infty}^{\infty} dx \ hw(x) C_{p}(x) = N. \text{ The expression for } C_{p}(x) \text{ does not have a simple analytic form, but it is straightforward to solve numerically.}

In Eq. (7), the diffusiophoretic flow velocity $v_{\text{ph}}(x)$ depends solely on the properties of the nanochannel, not the properties of the nanoparticle. So $v_{\text{os}}(x)$ can be determined from the known zeta potential of the channel walls or a prior measurement (Supplementary Note 1). Both the diffusion coefficient $D_{p}$ and the diffusiophoretic velocities $v_{\text{ph}}(x)$ depend on the diameter of the nanoparticle, and $v_{\text{os}}(x)$ also depends on its zeta potential $\zeta$. So a fit of Eq. (7) to an experimentally measured concentration profile has the particle diameter $d$ and zeta potential $\zeta$ as the only free parameters, except for an arbitrary scale factor that converts between the particle concentration and measured intensity. In all fits of concentration profiles presented below, the particle diameters, zeta potentials, and scale factors are fitted with no constraints between data sets.

Trapping of exosomes. We first demonstrate trapping, concentration and characterization of exosomes isolated from human blood serum of healthy donors. The stationary salinity gradients across the nanochannels are kept by a continuous flow of phosphate-buffered saline (PBS) through the microchannels. The concentrations are $C_{D}$ and $C_{W}$ at the narrow and wide ends of the nanochannels, respectively. Exosomes stained with the fluorescent dye DiO are introduced in the microchannel connected to the narrow ends of the nanochannels. At these ends of the nanochannels, diffusiophoresis dominates diffusiophoresis and particles migrate into the nanochannels and get trapped (Fig. 1e and Supplementary Movie 1).

After 90 s, 16 exosomes are trapped in the first nanochannel (see Fig. 1a). So, the particle concentration in the trap is ~400 times higher than the initial concentration in the microchannel (from 6 pM to 2.4 nM). Here the trap volume is defined as $V_{\text{trap}} = L_{\text{trap}}w(x_{0}) = 10.6 \mu L$. From the known flow rate in the microchannel, its cross-sectional area of 150 (μm)$^{2}$, and a particle concentration equal to 6 pM, we calculate that ~290 exosomes pass the entry of the nanochannel in 90 s. So the trapping efficiency of exosomes for a single nanochannel is 16/290 = 5.5%.
Figure 1e shows trapped exosomes at three different salinity gradients achieved by changing $C_N$ while maintaining $C_W$ at 10× PBS. Exosomes are trapped at physiologically relevant salinities (0.3–0.8× PBS), where the Debye length is ~1 nm (see “Methods”). The trapping positions and widths depend significantly on the gradient. For each measurement, a fit of the intensity profile gives the particle diameter and zeta potential, see Fig. 1f. Weighted averages over the three measurements at different salinity gradients (Fig. 1g) yields $d = (78 ± 7)$ nm for the particle diameter and $\zeta = (−18 ± 1)$ mV for the zeta potential. The latter is confirmed by LDE as $\zeta_{LDE} = (−20 ± 5)$ mV. Size measurements with DLS were inconclusive due to the polydispersity of the sample, but the measured size agrees with the peak in the size distribution provided by the vendor (Supplementary Note 3).
Fig. 4 Separating a mixture of liposomes. a Fluorescence image of trapped POPC:POPG 3:1 and POPC:POPG 1:3 liposomes marked with different fluorophores in a nanochannel (outlined with yellow). Experiment is performed at ln(C_W/C_W) = −8.1. Scale bar is 10 µm. b Same as a, but for liposomes with identical fluorophores. The image is an average over 10 s. Scale bar is 10 µm. c Fluorescence intensity of the two trapped populations shown in b (blue dots) and corresponding fit (red line) of the full distribution describing two particle populations, C_w(x) = w_1C_w,1(x) + w_2C_w,2(x). Here C_w,1 and C_w,2 are the fits to Eq. (7) for the two particle populations, and w_1 and w_2 are weight factors with w_1 + w_2 = 1.

Characterizing liposome ensembles. The trapping of exosomes demonstrates characterization of ensembles (Fig. 1). Here, we further validate the method with nine different combinations of liposome sizes and zeta potentials. We verify that results do not depend on the specific salinity gradient in the experiment, that all results are consistent with DLS and LDE measurement, and show how the trapping position x_0 depends on the liposome parameters and the salinity gradient.

Characterizing individual liposomes. Liposomes can be made with desired sizes and zeta potentials, so they are well suited for establishing trapping in a salinity gradient and to perform characterization. We use liposomes with three different lipid compositions, that is, different POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine):POPG (1-palmitoyl-2-oleoylsn-glycero-3-phosphoglycerol) ratios that are either 3:1, 1:1, or 1:3, and, consequently, different zeta potentials (−30, −36, and −48 mV). Liposomes were extruded through membranes with different pore sizes to produce subpopulations with different diameters (−70, −110, and −150 nm). Before loading the liposomes in the nanofluidic device, they were analyzed with DLS and LDE to benchmark our results.

We first measure the sizes and zeta potentials of individual liposomes. For a low concentration of liposomes in the microchannel, the filling rate of the trap is so slow that we can capture a single liposome in a nanochannel and track its stochastic motion for up to several minutes. So we load a nanochannel with a single liposome from an ensemble with a mean diameter d_{DLS} = (76 ± 3) nm and zeta potential ζ_{LDE} = (−28 ± 1) mV (Supplementary Movie 2). From microscopy images (Fig. 2a), we extract the time-dependent position of the particle in the nanochannel (Fig. 2b) and plot the x-coordinates (Fig. 2c).

We assume that in the long-time limit, the distribution of a single particle’s positions is identical to the spatial distribution for an ensemble of similar particles. So for each particle, we fit Eq. (7) to the histogram of its measured x-coordinates (black, dashed curves in Fig. 2d). For the histogram in the upper panel in Fig. 2d, the fit yields the particle diameter d = (72 ± 9) nm and the zeta potential ζ = (−30 ± 2) mV. This is consistent with the DLS and LDE measurements. Uncertainties are only ~15% and ~6%, respectively, of the measured values for a 40 s measurement. Other panels in Fig. 2d show histograms of x-coordinates from trajectories for two liposomes with different sizes (middle panel) and lipid compositions (lower panel). Notice how the changes in size and zeta potential alter the trapping positions x_0 and the widths of the distributions. Figure 2e shows fitted values for the diameters and zeta potentials for particles from the POPC:POPG 3:1 population with ζ_{LDE} = (−28 ± 1) mV and diameters d_{DLS} = (76 ± 3) nm (red, dashed lines). The measurements on individual liposomes indicate that particle-to-particle variation can be resolved by tracking individual particles in the nanofluidic trap.
Separating mixed liposome populations. For a fixed salt gradient, the distance between the trapping positions is only a few microns for the two largest particle sizes (d = 106 and 155 nm), much smaller than the widths of the spatial distributions of particles. The separation of trapping positions for different zeta potentials is significantly larger (see Fig. 3c, f). We therefore demonstrate separation of a liposome mixture based on surface characteristics and introduce an equal mixture of liposomes with identical sizes, but different lipid compositions, in the nanofluidic device. The liposomes are POPC:POPG 3:1 with diameters d\textsubscript{DLS} = (73 ± 3) nm and zeta potentials \( \zeta \text{DDE} = (28 ± 1) \text{ mV} \), and POPC:POPG 1:3 with d\textsubscript{DLS} = (73 ± 3) nm and \( \zeta \text{DDE} = (50 ± 3) \text{ mV} \). Figure 4a shows the separation of two liposome populations marked with different fluorophores (Supplementary Movie 3), where the separation is due to the different zeta potentials. Importantly, the separation is also clearly seen when both populations are marked with the same fluorophore (Fig. 4b). A fit to the particle distribution gives \( d = (75 ± 3) \text{ nm} \) and \( \zeta = (30 ± 1) \text{ mV} \) for POPC:POPG 3:1, and \( d = (69 ± 3) \text{ nm} \) and \( \zeta = (48 ± 2) \text{ mV} \) for POPC:POPG 1:3 (Fig. 4c). Results are consistent with DLS and LDE measurements, but the trapping method requires only a single measurement, and much smaller sample volumes and concentrations.

Discussion

From a single measurement lasting less than minutes, we can accurately determine both the particle size and zeta potential for individual particles, ensembles of particles, or even particle mixtures. Data analysis is based on a closed set of equations and does not require any calibration to simulations. Device fabrication by injection molding, a scalable industrial process, allows for low-cost mass production of the device and integration with microfluidics for sample and cell handling.

With its combination of rapid measurements and a single-use device, the technology can be applied to on-chip concentration of dilute samples in applications where sample amounts are scarce, for example, in single-cell analysis. By tuning the trapping position to physiological salinity, biochemical reactions, for example, immunoreactions, can occur and be monitored in real time. A novel device design may also allow for surface charge characterization of smaller particles, for example, individual proteins\(^2\), at physiological salinity. The method is equally viable for other types of nanoparticles, and the integration of this nanofluidic method with microfluidics would, for example, allow for in-line characterization of nanoparticle liquid phases synthesis. Finally, liquid biopsy-based diagnostics can be envisaged. For example, by taking advantage of the high concentrations of particles in the trap for label-tree detection of cancerous extracellular vesicles with Raman spectroscopy\(^3\).

Methods

Sample preparation. Purified exosomes derived from human blood serum in PBS (NaCl 137 mM, KCl 2.7 mM, Na\(_2\)HPO\(_4\) 10 mM, and KH\(_2\)PO\(_4\) 1.8 mM) were purchased from BioCat. The size distribution has its maximum at a diameter of 70 nm (full size distribution is shown in Supplementary Fig. 4). Exosomes were incubated at 37 °C with the green lipophilic fluorophore DiO. Excess fluorophores were removed by spin column purification. Liposomes were prepared by mixing the lipids POPC, POPG, and fluorophore (Texas red and DiO were used) at appropriate ratios and were dissolved in a 9:1 tertiary butanol to water solution. Samples were freeze dried overnight and the lipid film was rehydrated in PBS while being vortexed at 50 °C. All POPC:POPG mixtures were divided into three subpopulations, which were extruded through 30, 50, and 100 nm filters, respectively.

Mesodialysis contribution. The device was coated for 30 min with a phospholipid POPC:POPG mixture dissolved in 70% ethanol to passivate the surface. The ratio of the uncharged POPC to the charged POPG also determines the zeta potential of the channel walls \( \zeta \text{wall} \), and, consequently, the diffusioosmotic flow\(^3\). We used lipid coatings with a surface potential of \( \zeta \text{wall} = (−24 ± 1) \text{ mV} \) and \( \zeta \text{wall} = (−30 ± 1) \text{ mV} \) for the exosome and lysosome experiments, respectively. Characterization of the coatings is shown in Supplementary Note 1. To establish and maintain the salinity gradient in the nanochannels, PBS buffer solutions with two different concentrations were continuously flown through the microchannels by a 5 mbar pressure difference between the in- and outlets (Fig. 1a). Pressures were controlled by a Fluigent MFCs-EX pump with a stated instrumental error of ±0.3 mbar. The resulting flow rates in the microchannels are 450 pl min\(^−1\), and as the cross-sections of the microchannels are 150 (µm\(^2\)), the flow velocity is 50 µm s\(^−1\). Constant and reproducible residual flows of (36±15) fL min\(^−1\) were observed in the nanochannels with these pump settings (Supplementary Note 1). They correspond to a residual pressure across the nanochannel of 0.21 mbar, consistent with the instrumental error of the pump. The residual flows in the nanochannels were included in the data analysis by using \( v_\text{res}(s) = Q/(hw(s)) \) with the fitted value of Q, including the residual flow, shown in Supplementary Fig. 1b, rather than the theoretical expression for Q stated in and below Eq. (5). After the nanoparticles are introduced, it takes ~2 min before the salt gradient is established and is a sufficient amount of nanoparticles are trapped. The solution is then changed to one with identical salinity, but without nanoparticles. The data acquisition is then performed on the trapped particles. The fluorescence image from the trapped particles was recorded with a Nikon eclipse Ti2 microscope with a Photometrics Evolve 512 electron-multiplying charge-coupled device camera. A CoolLED pE-300 Ultra LED was used as light source.

Salt gradient in a funnel-shaped nanochannel. The salt concentration \( C(x) \) in the nanochannel depends on the salt concentrations in the two microchannels, that is, \( C_0 \) at \( x = 0 \) and \( C_M \) at \( x = L \) (see Fig. 1). The salt current density along the x-axis is \( J(x) = −D_0 \partial C(x)/\partial x \), where \( D_0 = (\zeta \text{ch} \text{DDE}/k_B T) \) is the Debye length, \( d \) is the diameter of the particle, \( \epsilon \) is the permittivity of the medium, \( \eta \) is the dynamic viscosity, \( Z \) is the valence of the solute, \( e \) is the elementary charge, \( u_t = 2\beta(F_i + F_c)/k_B T = 4\eta n/(1−y) \), and \( u_i = F_0 + \beta \lambda \), with \( F_i = \beta \lambda \) and \( F_c = \beta \lambda \). Here \( N_0 \) is Avogadro’s number, \( I \) the ionic strength, and \( P_e = \lambda \eta Z^2/(2\beta \epsilon) \) is the Peclet number. Furthermore, \( y = \tanh(\zeta/L) \), \( \zeta = Z(\epsilon/k_B T) \), \( \beta = (D_0 − D_2)/(D_2 + D_1) \), and \( D_0 = 2\eta D_2/(D_2 + D_1) \), where \( D_0 \) and \( D_2 \) are the diffusion coefficients of the cations and anions of a monovalent salt, respectively. The dominant ions in PBS are sodium ions, Na\(^+\), and chloride ions, Cl\(^−\), with the diffusion constants \( D_{Na} = 1330(\mu \text{m}^2 \text{s}^{-1}) \) and \( D_{Cl} = 2030(\mu \text{m}^2 \text{s}^{-1}) \), respectively\(^3\). Thus, \( \beta = −0.20 \) for NaCl. The F-functions depend on \( \zeta \) and are tabulated in Table 2 in ref. 28.

The diffusioosmotic mobility is \( M_{ph} = \lambda/(\lambda + \lambda e) \). So an expression for the diffusion coefficient of a particle in a nanochannel is required to determine the particle size and zeta potential from a concentration profile, see Eq. (7). We here use \( D_p = (1−1.004(d/h_0) + 0.418( dh_0)^2 + 0.21(d/h_0)^3 − 0.169(d/h_0)^4)/L^2 \), an expansion in the ratio between the particle diameter \( d \) and the channel height \( h_0 \) that is only strictly valid for a particle midway between two infinite walls. In Supplementary Note 2, we test experimentally the validity of the expression for our device and nanoparticles.

DLS and LDE. DLS and LDE measurements were performed with a Zetasizer Nano Z instrument to determine the nanoparticles’ diameters and zeta potentials, respectively.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Code availability

The code used in this study is available from the corresponding authors upon reasonable request.
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Acknowledgements
We thank J.B. Larsen for assisting with liposome synthesis.

Author contributions
R.M. and J.N.P. developed the project and led the experimental research. M.K.R. and R.M. performed the experiments. M.K.R. and J.N.P. developed the theory to predict the trapping and device behavior. M.K.R., R.M. and J.N.P. analyzed and interpreted the data. All authors co-wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-15889-3.

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Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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