Weighing nanoparticles in solution at the attogram scale

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Weighing nanoparticles in solution at the attogram scale

Selim Olcum\textsuperscript{a,b,1}, Nathan Cermak\textsuperscript{k}, Steven C. Wasserman\textsuperscript{a}, Kathleen S. Christine\textsuperscript{d,e}, Hiroshi Atsumi\textsuperscript{f}, Kris R. Payer\textsuperscript{g}, Wenjiang Shen\textsuperscript{h}, Jungchul Lee\textsuperscript{i}, Angela M. Belcher\textsuperscript{a,b,1}, Sangeeta N. Bhatia\textsuperscript{b,d,e,1,2}, and Scott R. Manalis\textsuperscript{b,c,1,2}

Departments of \textsuperscript{a}Biological Engineering, \textsuperscript{b}Materials Science and Engineering, \textsuperscript{c}Electrical Engineering and Computer Science, and \textsuperscript{d}Mechanical Engineering, \textsuperscript{e}Koch Institute for Integrative Cancer Research, \textsuperscript{f}Computational and Systems Biology Initiative, \textsuperscript{g}Harvard–MIT Health Sciences and Technology, \textsuperscript{h}Institute for Medical Engineering and Science, and \textsuperscript{i}Microsystems Technology Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139; \textsuperscript{j}Innovative Micro Technology, Santa Barbara, CA 93117; \textsuperscript{k}Department of Mechanical Engineering, Sogang University, Seoul 121-742, Korea; and \textsuperscript{l}Howard Hughes Medical Institute, Cambridge, MA 02139

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Physical characterization of nanoparticles is required for a wide range of applications. Nanomechanical resonators can quantify the mass of individual particles with detection limits down to a single atom in vacuum. However, applications are limited because performance is severely degraded in solution. Suspended micro- and nanochannel resonators have opened up the possibility of achieving vacuum-level precision for samples in the aqueous environment and a noise equivalent mass resolution of 27 attograms in 1-kHz bandwidth was previously achieved by Lee et al. [(2010) Nano Lett 10(7):2537–2542]. Here, we report on a series of advances that have improved the resolution by more than 30-fold, to 0.85 attograms in the same bandwidth, approaching the thermomechanical noise limit and enabling precise quantification of particles down to 10 nm with a throughput of more than 18,000 particles per hour. We demonstrate the potential of this capability by comparing the mass distributions of exosomes produced by different cell types and by characterizing the yield of self-assembled DNA nanoparticle structures.

Many aspects of engineered and naturally occurring aqueous nanoparticles with diameters below 50 nm remain unexplored. Particles in this size range play a central role in a wide range of applications, including targeted drug delivery (1, 2), therapeutic protein formulation (3, 4), and the study of intracellular signaling via exosomes (5). In all these cases, function is strongly correlated to particle size and concentration. Established methods for characterizing these particles such as electron microscopy, dynamic light scattering (DLS), and disk centrifugation can determine the size of particles down to the nanometer scale, but generally have limitations when it comes to heterogeneous samples, throughput, measuring concentration, or ease of use (6–8). Miniaturized resistive pulse sensors (9, 10) can quantify size, heterogeneity, and concentration of particles bigger than about 50 nm, but require high salinity, which is an important consideration when characterizing biological nanoparticles, such as protein aggregates.

Nanomechanical resonators in vacuum can characterize nanoparticles down to a single atom (11, 12) or protein (13, 14), but perform poorly when immersed in solution. Resonators with embedded fluidic channels, known as suspended micro- and nanochannel resonators (15–17) (SNRs and SMRs), exploit the extreme sensitivity of measurement in vacuum, while measuring particles in solution. Although performance of nanomechanical resonators in vacuum has been studied extensively (11, 12, 18–20), the practical detection limits of SNRs have only received theoretical treatment to date (21). A proof-of-concept SNR implementation detected gold nanoparticles with a buoyant mass of 77 attograms (ag) at low throughput (bandwidth) (17), far above the thermomechanical noise limit and insufficient to detect lighter particles of biological interest, such as exosomes. The performance achieved here approaches the thermomechanical noise limit, allowing us to measure the mass distributions of 10-nm gold particles and exosomes, which range in size from 30 to 100 nm (22).

Device Design and Evaluation

SNR systems work by measuring the resonant frequency of a microcantilever suspended in vacuum, which is extremely sensitive to changes in mass. A feedback loop keeps the cantilever oscillating at its resonant frequency while particles in solution flow through a U-shaped microfluidic channel running the length of the cantilever. As a particle passes through the cantilever, the cantilever mass transiently changes by the particle’s buoyant mass (particle mass minus mass of the fluid it displaces), inducing a brief detectable change in the oscillation frequency. Thus, the signal magnitude depends on the difference between the fluid density and the particle density, but all other solvent properties, such as salinity, can be varied depending upon the desired sample environment.

Improving SNRs to achieve attogram-scale resolution with this method requires increasing mass sensitivity and reducing frequency noise. Mass sensitivity is proportional to the resonant frequency of the cantilever and inversely proportional to its mass (23), so we designed and fabricated a family of SNRs with reduced masses and increased resonant frequencies (Table 1). The mass of the smallest cantilever design (type 3 in Table 1) is nearly 3× lower than previous designs (17) (type 0), with a resonant frequency nearly 5× greater, resulting in up to 14-fold sensitivity improvements. Moreover, frequency noise decreases as oscillation amplitude increases, until Duffing-type mechanical nonlinearity

Significance

Naturally occurring and engineered nanoparticles (e.g., exosomes, viruses, protein aggregates, and self-assembled nanostructures) have size- and concentration-dependent functionality, yet existing characterization methods in solution are limited for diameters below ~50 nm. In this study, we developed a nanomechanical resonator that can directly measure the mass of individual nanoparticles down to 10 nm with single-attogram (10\textsuperscript{-18} g) precision, enabling access to previously difficult-to-characterize natural and synthetic nanoparticles.


Conflict of interest statement: S.R.M. declares competing financial interests as a cofounder of Affinity Biosensors, which develops techniques relevant to the research presented. This article is a PNAS Direct Submission.

1S.O. and N.C. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: scottm@media.mit.edu.

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Table 1. Dimensions and theoretically calculated properties of the suspended nanochannel resonators

<table>
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<tr>
<th>Type</th>
<th>Length, μm</th>
<th>Thickness, μm</th>
<th>Width, μm</th>
<th>Channel height, nm</th>
<th>Channel width, μm</th>
<th>Resonant frequency, MHz</th>
<th>Stiffness, N/m</th>
<th>Mass, pg</th>
<th>Sensitivity, mHz/μg</th>
<th>δm(μg) (ag)</th>
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<td>50</td>
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<td>10</td>
<td>400</td>
<td>2</td>
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<td>3.5</td>
<td>1,059</td>
<td>−1.15</td>
<td>2.7</td>
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<tr>
<td>Type 1</td>
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<td>1</td>
<td>7.5</td>
<td>400</td>
<td>1</td>
<td>1.03</td>
<td>6.3</td>
<td>615</td>
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<td>1.2</td>
</tr>
<tr>
<td>Type 2</td>
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<td>1</td>
<td>7.5</td>
<td>400</td>
<td>1</td>
<td>1.99</td>
<td>16.9</td>
<td>443</td>
<td>−9.3</td>
<td>0.5</td>
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<tr>
<td>Type 3</td>
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<td>1</td>
<td>7.5</td>
<td>400</td>
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<td>29.1</td>
<td>369</td>
<td>−16.1</td>
<td>0.3</td>
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Properties were calculated assuming the cantilevers are filled with water. The thermomechanical limit of mass resolution (δm(μg)) is the Allan deviation of thermal energy-induced frequency fluctuations of the cantilever motion (28, 29) at gate time of 1 ms, when the cantilever is driven at the onset of mechanical nonlinearity (21) (SI Appendix, sections 3 and 4).

is observed (24). To reach optimal oscillation amplitudes, we used piezoceramic actuators to drive the cantilevers (Fig. 1A). Driving cantilevers beyond their linear range caused spring stiffening, which was indicated by a right shift of the open-loop frequency response curves (Fig. 1B). In this work, all resonators were driven at their onsets of nonlinearity to achieve the best frequency stability. It was not possible to drive resonators into this regime with the electrostatic actuation that was used in the previous systems (15, 17).

In the new SNR system (SI Appendix, Fig. S1), an optical lever setup similar to one previously described (17) detects the cantilever’s motion. The cantilever displacement signal acquired from a photodetector is phase-shifted via an adjustable delay on a field-programmable gate array (FPGA) and then amplified and fed back to a high-current amplifier driving a piezoceramic actuator. In the vicinity of the resonant frequency, intrinsic thermomechanical motion of the cantilever is the dominant source of noise on the photodetector output (SI Appendix, Fig. S2). The frequency of the oscillation is measured on the FPGA by digitally mixing the cantilever position signal down to 1 kHz and period counting using a 100-MHz clock.

To characterize the noise present in the frequency measurements of SNRs, we measured the oscillation frequency noise for different cantilevers (SI Appendix, Table S1) filled with ultrapure deionized water. We calculated the Allan deviations (25) (Methods) as a function of averaging (gate) times as shown in Fig. 2A, which is a common metric for oscillator noise. The Allan deviation of the overall oscillator system ranges from 4 to 8 parts per billion (ppb) at room temperature (without temperature control) using measurement rates of 5–1,000 Hz, which is the frequency range of interest for higher throughput. For type 2 and 3 cantilevers, this noise magnitude is equivalent to less than 1 ag (10⁻²¹ kg or 600 kilodaltons), which is demonstrated in Fig. 2B as mass-equivalent Allan deviation. The increasing noise at low gate times for type 0 and 1 cantilevers corresponds to white frequency noise, the flat region at the center for all cantilever types corresponds to the flicker (1/2) frequency noise and the ramp in the higher averaging durations corresponds to Brownian frequency noise and long-term frequency drift of the oscillators (26, 27).

To quantify the potential for further reductions in the noise level, we calculated the ultimate limit of frequency stability imposed by intrinsic thermomechanical fluctuations (28, 29) (SI Appendix, sections 3 and 4) for resonators driven at their onset of nonlinearity (21) (dashed lines in gray region of Fig. 2A). Measured frequency stability values at 1-ms gate time are 1.8- to 3.4-fold...
above the thermomechanical noise limits. For shorter (<10 to <1 ms) gate times, noise from the photodetector becomes the dominant factor (SI Appendix, Fig. S3), which sets a lower bound on the particle transit time and hence an upper bound on the throughput of the device. With further improvement of the detection system, it may be possible to achieve a 10-fold improvement in the throughput without sacrificing mass resolution (SI Appendix, section 3).

After we achieved mass sensitivities exceeding 16 mHz/ag and reduced frequency noise to 4 ppb, we focused on optimizing our peak detection scheme. We used the characteristic shape of a peak (30), determined by the flow path and the transit time of the particle, in a bank of matched filters for detecting the characteristic frequency modulation signal due to a particle transit with the maximum signal-to-noise ratio (31). The mass equivalent Allan deviation of type 2 and 3 devices is between 0.75 and 1.5 ag (gray region in Fig. 2B) and decrease as 1/(averaging time) (31). The results show 3σ detection limits lower than 5 ag or 3 MDa (SI Appendix, Figs. S4 and S5).

Results

Mixture of Gold Nanoparticles. Mass distribution is an important measure of nanoparticle populations. We first demonstrated the mass resolution of our system by weighing a mixture of 10-, 15-, and 20-nm gold nanoparticles. Before analyzing the mixture, we calibrated the mass sensitivity (Hz/kg) of the resonator using size-calibrated gold nanoparticles (SI Appendix, Fig. S6). In a 97-min experiment, we measured more than 29,000 individual particles in the mixture (Fig. 3A and B). The results show three distinct well-separated populations (Fig. 3C) ∼9.1, 28.1, and 73.4 ag. Assuming all particles are spherical and uniformly dense, the mean sizes of the three populations are estimated to be 9.9, 14.4, and 19.7 nm, which agree well with the manufacturer specifications of 9.9, 14.3, and 20.4 nm (Fig. 3D). The coefficients of variation in diameter for each population were 7.4%, 5.3%, and 4.9%, respectively, compared with the datasheet values of <8% for 15- and 20-nm gold nanoparticles. Additionally, we compared our results to DLS measurements (SI Appendix, Table S2), which could not resolve the three populations separately in the gold nanoparticle mixture. We also tested the dynamic range of the SNRs by successfully weighing larger particles (150-, 200-, and 220-nm polystyrene beads) using the same operating, detection, and estimation conditions (SI Appendix, Fig. S7).

In addition to mass distribution, concentration is also a key parameter of nanoparticle suspensions. The SNR can provide a direct measure of nanoparticle concentration because the detection and estimation algorithm estimates the transit time of each particle (Fig. 3B) and the dimensions of the buried microfluidic channel are known. Based on the measurement shown in Fig. 3, the concentrations of 10-, 15-, and 20-nm gold nanoparticles in the mixture are $5.4 \times 10^9$, $3.6 \times 10^9$, and $3.7 \times 10^9$ particles per milliliter, respectively (see SI Appendix, section 9, for error analysis), which are comparable with the concentrations obtained from the particle datasheets ($5.7 \times 10^9$, $3.1 \times 10^9$, and $3.9 \times 10^9$ particles per milliliter).

Heterogeneity of Exosomes from Different Cell Types. To demonstrate the capability of the SNRs to characterize relevant biological samples, we used exosomes, which are cell-derived vesicles present in the extracellular fluids that mediate intercellular communication via the exchange of proteins and genetic material (32, 33). Although there is immense scientific and clinical interest, detection and characterization of exosomes remain challenging. Purified exosomes from in vitro and clinical samples alike are heterogeneous because their size and density ranges from 30 to 100 nm and 1.13 to 1.19 g/cm³, respectively (22), which translates into 2–100 ag buoyant mass in water. Moreover, exosomes from a mixed population of cells, i.e., normal vs. diseased cells, theoretically can differ in their cargo content, which in turn may alter their mass, size, and/or density. Optical methods such as DLS analysis can give comparative information about their mean size (SI Appendix, Table S3), but the heterogeneity and distribution shape of the populations, which may reflect differences in their biological functions, are difficult to measure. Therefore, we weighed exosomes that were produced by 3T3-J2 fibroblasts and primary hepatocytes, two inherently different cell types, which when cocultured have been shown to engage in both physical and molecular cell–cell interactions (34, 35). The buoyant mass distributions of exosomes derived from fibroblast and hepatocyte cells reveal clear differences in heterogeneity (Fig. 4). The relative broadness in buoyant mass of the fibroblast exosomes suggests the presence of either a larger or denser subpopulation compared with the exosomes derived from hepatocytes.
Buoyant mass measurements of exosomes derived from different cell types. Buoyant mass distributions (kernel density estimates) of fibroblast-derived (red) and hepatocyte-derived (black) exosomal vesicles. Some 7,100 fibroblast exosomes and 9,600 hepatocyte exosomes are weighed using an SNR in 65-min and 76-min experiments, respectively. The limit of detection is depicted with a vertical dotted line close to 5 ag. (Inset) Estimation of exosome diameter by assuming a spherical shape and a constant exosome density of 1.16 g/mL throughout the populations. The vertical dashed line indicates the corresponding limit of mass detection as 39 nm.

Fig. 4. Buoyant mass measurements of exosomes derived from different cell types. Buoyant mass distributions (kernel density estimates) of fibroblast-derived (red) and hepatocyte-derived (black) exosomal vesicles. Some 7,100 fibroblast exosomes and 9,600 hepatocyte exosomes are weighed using an SNR in 65-min and 76-min experiments, respectively. The limit of detection is depicted with a vertical dotted line close to 5 ag. (Inset) Estimation of exosome diameter by assuming a spherical shape and a constant exosome density of 1.16 g/mL throughout the populations. The vertical dashed line indicates the corresponding limit of mass detection as 39 nm.

from hepatocytes, which shows less dispersion with a 35% coefficient of variation in mass and a median of 8.6 ag; this translates into a median diameter of 48 nm (Fig. 4, Inset), assuming a spherical shape and uniform exosome density of 1.16 g/mL (see SI Appendix, Fig. S9 for the effect of density assumption on size). We observe a rapid increase in the exosome concentrations in both populations with decreasing buoyant mass from 20 to 10 ag. Similar size distributions have been measured using transmission electron microscopy (TEM) previously (36) on other types of exosome samples. It is worth noting that the shape of the distribution below ~7–8 ag is uncertain, because what we observe in this region is predominantly defined by the detection probability (SI Appendix, Fig. S6C). We repeated the experiments using a different cantilever on the same samples as well as on a second batch of purified fibroblast and hepatocyte exosomes. The results of the repeated runs (SI Appendix, Fig. S10) suggest the same difference in population heterogeneity presented in Fig. 4. We determined the concentrations of the purified stocks as being $3.3 \times 10^{12}$ and $1.3 \times 10^{12}$ particles per milliliter for fibroblast and hepatocyte exosomes, respectively, demonstrating that this technique can be used to quantify yields of exosome purifications as well.

Yield of DNA Origami–Gold Nanoparticle Assemblies. To further demonstrate the absolute concentration measurement capability of the SNR, we characterized the binding efficiency of functionalized gold nanoparticles to DNA origami structures. DNA nanotechnology has great promise for developing precise nanostuctures, such as scaffolds for molecular nanodevices (37). However, practical and accurate methods are required for assessing the yield of complex DNA structures. We designed DNA origami structures as scaffolds with two and three binding sites for gold nanoparticles (SI Appendix, Figs. S11 and S12) and validated the binding of gold nanoparticles to DNA origami by gel electrophoresis and atomic force microscopy (AFM) (SI Appendix, Fig. S13). We weighed ssDNA-modified 15-nm gold nanoparticle binding agents with the SNR and observed that ~13% of the DNA-modified gold nanoparticle population were not singles (as defined as weighing more than 45 ag), with only 3.3% of the population being above 75 ag ($9 \times 10^7$ particles per milliliter). This population results from two or more particles that agglomerated due to nonspecific binding of the modified DNA. We then weighed the DNA origami structures with two binding sites with modified gold nanoparticles. The abundance of the nonsingles increased to 32%, with 9% of this fraction weighing above 75 ag ($1.3 \times 10^8$ particles per milliliter). This increase in the nonsingles indicates the successful binding of the gold nanoparticles to the DNA origami structure. Finally, we weighed the DNA origami structures with three binding sites with modified gold nanoparticles and, as expected, observed a broader distribution of particles compared with the previous samples. We calculated the concentration of the particles that are heavier than 75 ag...
as $4.1 \times 10^8$ particles per milliliter, which constitute about 47% of the population. The resulting buoyant mass distributions for the three sample populations are shown in Fig. 5. Although gel electrophoresis can provide similar distributions, it cannot provide a measure of absolute concentration. Because the flow rate through the SNR in this experiment was ~3 nL/h, we envision that SNRs could ultimately be used as a real-time tool to quantify nanostructure assemblies.

Outlook

The demonstrations presented in this paper suggest that the SNR can be a valuable complement to the existing methods for characterizing nanoparticles in solution. High-precision mass measurement could allow us to identify signatures of pathology in blood plasma regardless of the molecular properties of the target. For example, glioblastoma cells are known to secrete microvesicles (50–500 nm) that have been implicated in angiogenesis (32). However, assessing if such nanoscale vesicles display a unique size or concentration signature has previously been extremely difficult. Combining volume measurements via resistive pulse sensing and buoyant mass measurements via the SNR at the level of individual nanoparticles would reveal their density, further increasing potential diagnostic power. For exosomes, measuring density would enable small particles with high amounts of cargo to be distinguished from large particles with limited cargo. Moreover, the SNR can potentially be used to discriminate between exosomes and larger extracellular microvesicles, which differ in size (38) and potentially differ in terms of their function. Because the contents of microvesicles and exosomes remain poorly characterized, multiparameter physical measurements together with molecular measurements could help elucidate their biological functions. In addition, future SNR implementations incorporating particle sorting and collection could allow purification and downstream analyses on a range of biological and synthetic nanoparticle populations. Such SNR implementations could be used for monitoring nanoparticle formation kinetics and ultimately for improving the techniques for engineering synthetic nanoparticles with desired properties.

Methods

SNR Fabrication. SNRs were manufactured by a previously described process (15, 17), which was performed at Innovative Micro Technology. The process enables each cantilever to freely oscillate in a dedicated vacuum cavity with an on-chip getter to maintain the high vacuum required for the high-Q operation. There are four fluidic ports drilled on the top glass wafer to access the two bypass channels ($50 \times 20 \mu m$) separated 285 $\mu m$ apart at each side of the cantilever. The U-shaped channel in the cantilever is connected to these bigger bypass channels by 140-$\mu m$ channels with the same cross-section that is in the cantilever.

System Operation. The displacement noise of the cantilever due to the thermal energy is amplified in a positive feedback loop to achieve a sustainable self-oscillation according to the Barkhausen criteria (39) at the instantaneous resonant frequency of the cantilever. The frequency of oscillation is measured by period counting at 100 MHz using a digital heterodyne mixer and a low-pass filter coded in the FPGA (SI Appendix, section 2). We use computer-controlled electronic pressure regulators connected to pressurized glass sample vials to control the flow in the bypass channels and in the SNR.

Allan Deviation. The Allan deviation, $\alpha_A(\tau)$, of the oscillation frequency of an oscillator in a time period of $\tau$ is defined as in ref. 40:

$$\alpha_A(\tau) = \sqrt{\frac{\sigma_f(\tau)^2}{\tau}} = \sqrt{\frac{1}{2(N-1)} \sum_{i=1}^{N} \left(\frac{\tau_i - \tau_i-1}{\tau_i}\right)^2},$$

where $\tau_i$ is the time average of the frequency measurement in the $k$th time interval of $\tau$ within a total of $N$ intervals, and $\tau_0$ is the mean oscillation frequency calculated over the entire duration of the noise measurement. In other words, the Allan deviation is calculated by averaging subsequent sections of the normalized frequency data of length $\tau$, and then taking the difference between the means of contiguous segments.

Peak Detection and Estimation. Measured frequency data by the FPGA is sent to a control computer in real time via Ethernet and is recorded by the computer. The recorded data are analyzed afterward using postprocessing code in Matlab. First, the mean of the data are subtracted and the result is high-pass filtered by a second-order IIR notch filter with 1-Hz cutoff frequency. Next, the data are filtered with a bank of matched filters (SI Appendix, Fig. S4) with coefficients having a shape of a frequency peak (30) resulting from a particle passing through the SNR. The widths of the filters in the bank are adjusted to span the possible transit times of the particles for the computer. The recorded data are filtered and sampled in parallel, and the signal-to-noise ratio is calculated by a least-squares fit algorithm.

Calibration. The mass sensitivity of a cantilever is determined by running a population of gold nanoparticles [RM 8012 by National Institute of Standards and Technology (NIST)] calibrated as a reference material before the actual experiment. Cantilever sensitivity is calculated using the mean particle diameter (26.5 nm), which was estimated by AFM, SEM, and TEM measurements described in the reference material dataset. The resulting mass histogram and size estimation are given in SI Appendix, Fig. S6.

Gold Nanoparticle Measurements. A type 2 device (11M in SI Appendix, Table S1) was used to weigh the nanoparticle mixture comprised of 10– (NIST RM 8011), 15–, and 20-nm (EMGC15 and EMGC20 from BBI Solutions) gold. Samples were diluted in filtered (0.22 µm) water 300, 150, and 60 times, respectively. We mixed 0.5 mL of diluted 10-, 15-, and 20-nm gold particles together before the experiment, which increased the total dilutions to 900, 450, and 180 times, respectively. The accuracy of the concentration estimation increases with the signal-to-noise ratio of the particles in the sample (SI Appendix, section 9). Therefore, we calculated flow rate in the SNR using the 20-nm particle signal, which has the highest signal-to-noise ratio in the mixture.

Functionalization of Gold Nanoparticles with DNA. DNA-modified gold nanoparticles (SI Appendix, Fig. S11) were prepared using previous reports (41). Gold nanoparticles (15 nm; Ted Pella Inc.) were stabilized with Bis(p-sulfonatophenyl)phenylphosphine dihydrate dipotassium salt (BSPP). BSPP (18 mg) was dissolved in gold nanoparticle solution (25 mL), and the mixture was stirred overnight at room temperature. Sodium chloride was added slowly to the solution with stirring until the color changed from red to light purple. The resulting solution was centrifuged at 366 × $g$ for 30 min. The supernatant was carefully removed and the gold nanoparticles were dispersed in 0.5 mL BSPP (2.5 mM) with 0.5 mL methanol. The solution was centrifuged at 21,130 × $g$ for 30 min. The supernatant was removed and gold nanoparticles
were dispersed again in 0.25 mL BSSP (2.5 mM). Next, 80 µL T2-pS-H DNA (100 nM) was activated with 20 µL Tris-(2-carboxyethyl)phosphine hydrochloride (100 mM). DNA-activated thiol-modified DNA was purified using a NAP-5 column (GE Healthcare). The phosphonated gold nanoparticles and thiol-modified DNA (gold nanoparticle:DNA = 1:200) were incubated in 1 M Tris, 0.9 M boric acid, 0.01 M EDTA (pH 8.0) containing NaCl (50 mM) for 15 h at room temperature, which enabled the stabilization of gold nanoparticles under high-salt conditions.

Preparation of the DNA Origami. The molar ratio of 1:5 between M13mp18 viral ssDNA and staple strands was used. DNA origami was assembled in 40 mM Tris, 20 mM acetic acid, 2 mM EDTA (pH 8.0) buffer (1:10) containing magnesium acetate (12.5 mM) by annealing from 95 °C for 5 min to 60 °C over 35 min, and cooled further to 15 °C over 135 min. The annealing process was purified using spin filtration (MWCO, 100 K; Millipore) to remove extra staple strands. The constructed DNA origami was examined by AFM (SI Appendix, Fig. S12). DNA origami structures with two and three binding sites were prepared by substituting original staple strands for hook staple strands. Purified DNA origami was mixed with thiol-modified gold nanoparticles and annealed from 37 °C to 15 °C over 110 min. Finally, we examined the products purified by agarose gel by AFM (SI Appendix, Fig. S13).

Exosome Experiments. Exosomes were purified from supernatants of 3T3-L2 fibroblasts cultured at 37 °C, 5% CO2 in DMEM with high glucose, 10% (vol/vol) exosome-depleted FBS, and 1% penicillin–streptomycin. 3T3-L2 fibroblasts were cultured until ~60% confluency at which point media was replaced with exosome-depleted fibroblast media. After 48 h, 3T3-L2 media was collected and centrifuged at 1,800 × g for 10 min to remove cells. Hepatocytes exosomes were obtained from primary rat hepatocytes cultured in DMEM with high glucose, 10% (vol/vol) exosome-depleted FBS, 0.5 U/mL insulin, 7 ng/mL glucagon, 7.5 µg/mL hydrocortisone, and 1% penicillin–streptomycin. Twelve million hepatocytes were seeded in T150 flasks for 3 h to obtain ~80% confluence. After 3 h, media was collected, centrifuged at 3,000 rpm for 10 min to remove cells, and replaced with fresh exosome-depleted hepatocyte media. Following an additional 24 h of culture, hepatocyte media was again collected, centrifuged at 3,000 rpm for 10 min to remove cells, and pooled with hepatocyte media collected the previous day. Exosomes were purified from media using differential centrifugation. Briefly, media was centrifuged at 10,000 × g for 1 h and subsequently processed through a 0.22-µm filter. A crude exosome pellet was obtained by ultracentrifugation at 100,000 × g for 3 h at 4 °C and resuspended in 0.22 µm filtered PBS. Washed exosomes were again pelleted at 100,000 × g for 2 h at 4 °C and resuspended in 150 µL filtered PBS. To remove any copurified protein complexes, exosomes were further purified by size-exclusion chromatography over a Sepharose CL-4B resin column (GE Healthcare). Exosome containing fractions, as detected by DLS analysis, were collected and pelleted at 100,000 × g for 2 h at 4 °C. Finally, purified exosomes were resuspended in 100 µL filtered PBS for further analysis. We diluted the prepared fibroblast exosomes 500× and hepatocyte exosomes 200× in 0.22 µm filtered 1× PBS before running the populations through a type 3 SNR (see 7B in SI Appendix, Table S1).

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