Acoustic Sensing Based on Density Shift of Microspheres by Surface Binding of Gold Nanoparticles

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Herein, we propose a concept for sensing based on density changes of microparticles (MPs) caused by a biochemical reaction. The MPs are levitated by a combined acoustic–gravitational force at a position determined by the density and compressibility. Importantly, the levitation is independent of the MPs sizes. When gold nanoparticles (AuNPs) are bound on the surface of polymer MPs through a reaction, the density of the MPs dramatically increases, and their levitation position in the acoustic–gravitational field is lowered. Because the shift of the levitation position is proportional to the number of AuNPs bound on one MP, we can determine the number of molecules involved in the reaction. The avidin–biotin reaction is used to demonstrate the effectiveness of this concept. The number of molecules involved in the reaction is very small because the reaction space is small for an MP; thus, the method has potential for highly sensitive detection.

Keywords Acoustic levitation, reactions on a microsphere, Au nanoparticles, avidin–biotin reaction, density change

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Introduction

Microparticles (MPs) and nanoparticles (NPs) have received increasing attention because they allow flexible designs of analytical methods, particularly in bioanalyses and bioassays. A number of methods utilizing the characteristic natures of MPs and NPs have been proposed. The particles used for such purposes are classified into several categories according to the size and material, e.g., metal NPs, quantum dots, magnetic NPs (MagNPs), mineral MPs, and polymer MPs. Metal NPs—such as gold NPs (AuNPs) and silver NPs—are most extensively employed because various sizes and shapes are available, their surface can be biologically or chemically modified in relatively simple ways, and they often exhibit characteristic colors originating from plasmon absorption. AuNPs usually have a reddish color, and their absorption wavelength strongly depends on the particle size. Therefore, if they are aggregated as the result of a reaction with mediators, the mediators can be detected according to any color change, even with the naked eyes. The detection of DNA and proteins has been performed using this phenomenon. The aggregation of metal NPs has also been applied to surface-enhanced Raman scattering (SERS) and light-scattering detection.

The manipulation and separation of particles play key roles in the successful implementation of particle-based bioanalyses. The magnetic nature of the particles allows easy manipulation or separation because they are attracted or repelled by a magnet. The trapping of MagNPs in a desired location facilitates the sophisticated design of analysis schemes; e.g., rinsing after a reaction or consecutive reactions with different reagents is easily performed for the entrapped particles. Other physical fields, including electric, dielectric, flow, optical, and acoustic fields, have also been employed for particle manipulation and separation. In some cases, these fields are highly adaptable to microfluidic devices in which many elemental steps required for entire analyses—e.g., target recognition, reactions, separation, and in some cases detection—can be integrated.

Hence, most of the key steps required for particle-based analyses, including particle preparation, manipulation techniques, and combination with microfluidic devices, have been well developed. However, detection strongly relies on conventional methods, mostly spectrometry. For example, fluorescence measurements are extensively applied and exhibit high sensitivity. Bound-free separation is crucial for high sensitivity but is often a difficult and time-consuming process in the entire analytical procedure. Although SERS is also a powerful method, its utilization for quantification has not been well established, because of its insufficient repeatability and reliability. Thus, one of the current bottlenecks for particle-based bioanalyses is the limited availability of efficient detection methods that can indicate the biochemical reactions occurring on the particle surface as a measurable quantity.

We developed a combined acoustic-gravitational field for the separation and characterization of MPs. The levitation coordinate of a particle in this field is a function of the density and compressibility of the particle. If these acoustic properties of a particle are altered as a result of a reaction, the levitation coordinate changes accordingly. This principle allowed us to study single-bead ion-exchange, which causes changes in the density and compressibility of the resin bead. In this case, the progress of the ion-exchange reaction (ion recognition) is
converted into the levitation coordinate, which is a measurable quantity. The reactions, which cause large coordinate changes, are desirable for high sensitivity.

In the present study, the avidin-biotin reaction is utilized as the binding between polymer MPs and AuNPs, and the levitation of the former is studied under various conditions. The density of gold is approximately 15-times higher than that of the polymer MPs. Therefore, even though a small number of AuNPs are bound on the polymer MPs, the levitation of the MPs undergoes significant modification. We present the efficiency of this concept and its great potential for sensitive and versatile analyses, particularly bioanalyses.

**Experimental**

**Instruments**

The experimental setup for acoustic-levitation studies is shown in Fig. S1 (Supporting Information). A transducer (2 cm × 2 cm lead zirconate titanate, resonance frequency of 500 kHz, Fuji Ceramics) was driven by sinusoidal signals generated by a function generator (Model WF1946, NF Electric), and was amplified by a bipolar high-speed amplifier (Model 4015, NF Electric). The transducer was set on a three-dimensional stage to adjust the vertical and horizontal positions. The particle behavior was observed using a CCD camera (Model CS220, Olympus) through a zoom lens (maximum magnification of ×24). The relative coordinates were determined using digital images. The size of the pixels on the images was calibrated by vertically moving the x-stage; one pixel corresponded to 0.46 μm. The levitation coordinate was determined from a digital image using this pixel size.

A fused silica cell (30 mm in length, 8 mm in width, and 12.62 mm in height) with a rectangular through-channel (3.0 mm in width and 1.50 mm in height) was used. The cell wall thickness (5.56 mm) and the channel height (1.50 mm) were equal to the half-wavelengths of 500-kHz ultrasound in silica glass and water, respectively. Thus, the node of the standing wave should be formed at the center of the channel filled with water. The cell was pasted on a transducer by using nail enamel as an adhesive. Because the resonance frequency depended on the instrument setup and the experimental conditions, the frequency was optimized daily to ensure stable levitation of the particles.

Acrylic particles and gold-plated particles (10 μm in diameter) were purchased from GreenChem Inc. (Osaka, Japan). Biotin-bonded AuNPs were purchased from Cytodiagnostics (Burlington, Canada).

The density of the MPs was determined via heavy liquid separation by using sodium polytungstate (SPT) solutions with various densities as media.21 The densities of the SPT solutions were determined via gravimetry. Figure S2 (Supporting Information) shows the relationship between the density and the concentration of the SPT solutions.

**Preparation of epoxy resin**

A membrane emulsion method was used to prepare epoxy particles (EPs).33 Ethylene glycol dimethacrylate and glycidyl methacrylate were mixed at a ratio of 4:1. Then, 1 w/v% 2,2-azobisisobutyronitril) was added to this organic phase as a polymerization initiator. The mixed organic phase was added to a 0.2% aqueous methyl cellulose solution through a porous silica membrane (pore size, 3 μm) with stirring. The drops formed on the membrane surface were detached by a shea flow of the aqueous phase. The dispensation rate of the organic phase was controlled by a syringe pump (Model Pump 11, Harvard Apparatus), and the typical flow rate was 0.025 mL min⁻¹. The optimum stirring rate was determined to be 400 rpm, according to the size and monodispersity of the formed emulsions. The resulting emulsions were heated at 78°C for 4 h to induce polymerization. The formed EPs were collected via centrifugation and washed with deionized water and ethanol. Figure S3 (Supporting Information) shows the size distribution of the EPs, which was determined via microscopic observation. The average diameter of the EPs was 4.4 μm. The broad size distribution, which is not desirable for measurements based on a single microsphere, is not a serious issue in the present study because the particle behavior as an aggregate was evaluated.

**Preparation of AuNP-bound EPs**

The EP MSs were modified with avidin, as schematically shown in Fig. S4 (Supporting Information). We added 2.64 mg of the EPs to an aqueous solution of avidin (3.02 μM) prepared in a borate buffer (pH 9.18); the mixture was then shaken for completion of the surface reaction. Epoxide groups on the surface of the EPs reacted with amine groups in the avidin molecules under a basic condition. The avidin-modified EPs (avEPs) were washed with deionized water and dried at a reduced pressure. The number of avidin molecules on the surface of the EPs was determined using the reaction with biotin-4-fluorescein. The number of avidin molecules bonded on each EP was 9.44 × 10⁴.34,35 The number of AuNPs bound to the avEPs was controlled by changing the ratio of AuNPs to avEPs. The reaction between the avEPs and bAuNPs caused the avEPs to appear reddish. The AuNP-bound EPs were collected via centrifugation to remove any unreacted AuNPs.

**Results and Discussion**

Particle behavior in an acoustic–gravitational field

According to Yoshioka and Kawasima,37 the ultrasound radiation force imposed on a particle (\( F_u \)) is given as

\[
F_u = \frac{8\pi}{3\lambda} r^3 E_u A \sin \left( \frac{4\pi z}{\lambda} \right),
\]

\[
A = \frac{5\rho' - 2\rho + \gamma'}{2\rho + \rho - \gamma},
\]

where \( r \) is the radius of the particle; \( \lambda \) is the ultrasound wavelength, \( E_u \) is the average ultrasound energy density, \( z \) is the distance from the node of the ultrasound standing wave (defined as the levitation coordinate, \( z = 0 \) at the node), \( \rho \) and \( \gamma \) are the density and compressibility of the medium (water in the present case), respectively, and the single quotation mark represents the corresponding properties of the particle. When \( A \) is positive, the ultrasound radiation force moves the particles towards the node of the standing wave. Therefore, Eq. (1) predicts that all particles are levitated at the node, regardless of their physical properties, as long as \( A \) is positive. This principle has been utilized for various applications, such as the aggregation and trapping of particles—including biological cells—and the separation of particles.71,18
When an ultrasound standing wave is vertically formed, the particles simultaneously undergo the sedimentation force and ultrasound radiation force. They are levitated at the equilibrium position, where the acoustic radiation force and sedimentation force are balanced. The levitation coordinate, \( z \), is given as

\[
z = \frac{\lambda}{4\pi} \sin^{-1} \left( \frac{(\rho - \rho') g \lambda}{AE_{ac} 2\pi} \right),
\]

where \( g \) is the gravitational acceleration. Eq. (3) suggests that \( z \) is independent of the particle size, but is a function of the particle density and compressibility. Thus, we can resolve these properties of a particle according to the difference in the levitation coordinate in the combined acoustic–gravitational field.

**Behavior of gold-plated microspheres in an acoustic–gravitational field**

The levitation behavior of gold-plated acrylic MPs (AuPAMs) in an acoustic–gravitational field was investigated. AuPAMs having different gold-layer thicknesses—\( d = 30, 50, 70, \) and 90 nm—were examined. The data for \( d \) were taken from the product information. The densities of the microspheres measured via the method described in the experimental section are listed in Table 1. Using \( d \), the density of the AuPAMs can be calculated as

\[
\rho' = \frac{\frac{4}{3} \pi r^3 \rho_{AM} + \frac{4}{3} \pi (r + d)^3 - r^3 \rho_{Au}}{\frac{4}{3} \pi (r + d)^3},
\]

where \( \rho_{AM} \) and \( \rho_{Au} \) (= 19.32 g cm\(^{-3}\)) are the densities of the base acrylic MP (AM) and gold, respectively. The densities calculated using Eq. (4) are also listed in Table 1. The measured values agree well with the calculated ones. Thus, these particles of known density were used for assessing the particle behavior in the present field.

Figure 1A shows photos of levitated AMs and AuPAMs with \( d = 50 \) and 90 nm. In the present measurements, several tens of particles were simultaneously introduced into a cell. The particles were captured by the lateral acoustic force, and formed an aggregate, as shown in the photos. The levitation coordinate was defined as the average of the upper and lower edge coordinates of the aggregation band. Obviously, heavier particles (with a thicker gold layer) were levitated at lower positions. Figure 1B shows the dependence of \( z \) on the voltage applied to the transducer (\( V \)); \( V^2 \) is proportional to \( E_{ac} \). As \( V \) decreases, the acoustic force decreases, and \( z \) becomes negative. When the acoustic force becomes smaller than a threshold value, particles are no longer levitated, but settle down on the bottom of the cell. As the gold layer becomes thicker, the \( z \) measured at a particular \( V \) decreases, mainly because of the increased density. The curves shown in Fig. 1B are the results of curve-fitting based on Eq. (3), where the compressibility was used as a fitting parameter. The compressibility for the AMs was determined to be 2.30 \( \times 10^{-10} \) Pa\(^{-1}\). The compressibility of a composite particle \( (\rho') \) is given by

\[
\gamma' = \phi_1 \gamma_1 + \phi_2 \gamma_2,
\]

where \( \phi_1 \) and \( \phi_2 \) are the volume ratios for the two components (AM and gold in this case), and \( \gamma_1 \) and \( \gamma_2 \) are the compressibility values for the two components. The compressibility of gold is reported to be 5.88 \( \times 10^{-12} \) Pa\(^{-1}\). Equation (5) predicts that the decrease in \( \gamma' \) with increasing \( d \) is so small (\( \gamma' = 2.18 \times 10^{-10} \) Pa\(^{-1}\) for an AuPAM with \( d = 90 \) nm) that its impact on \( z \) is

| \( d/\text{nm} \) | \( \rho' \) measured/g cm\(^{-3} \) | \( \rho' \) calculated/g cm\(^{-3} \) |
|---|---|---|
| 0 | 1.265 | — |
| 30 | 1.560 | 1.59 |
| 50 | 1.775 | 1.80 |
| 70 | 1.948 | 2.00 |
| 90 | 2.160 | 2.21 |
negligible; thus, the difference in the levitation coordinate between the AMs (as a reference) and AuPAMs ($\Delta z$) is mostly determined by the density change due to the different gold-layer thicknesses. Figure 2 shows a linear relationship between $d$ and $\Delta z$ measured at $V = 6.25$ and 7.50 V. Equation (4) predicts a linear relationship between $\rho'$ and $d$ for $r >> d$. In such cases, Eq. (3) predicts an almost linear relationship between $\rho'$ and $\Delta z$, and eventually between $d$ and $\Delta z$. Thus, the linear relationships shown in Fig. 2 agree with the theoretical predictions, and suggest that the amount of gold on the MPs can be determined according to $\Delta z$.

Change in z induced by AuNP binding through the avidin-biotin reaction

The levitation coordinate of the MPs is changed by the density increase caused by the binding of AuNPs to the MPs, as schematically shown in Fig. 3. The avidin-biotin reaction occurring on the MP surface was examined as a model system. The aforementioned results suggest that we can determine the number of reactions occurring on an MP according to $\Delta z$. For this purpose, EPs were synthesized, and avidin was introduced on their surface. The prepared EPs were characterized, as summarized in Table 2.

Figure 4 shows the calculated $\Delta z$ with respect to the number of AuNPs bound on one EP. The diameter of each EP was assumed to be 4.4 $\mu$m, which is equal to the average diameter of the synthesized EPs. Two different sizes of AuNP (50 and 100 nm) were assumed. Figure 4 predicts that the binding of 100-nm AuNPs yields a larger $\Delta z$ than that of 50-nm AuNPs, suggesting that a higher sensitivity is achieved when larger AuNPs are used. Additionally, $\Delta z$ is almost proportional to the number of AuNPs bound on each EP, indicating that the AuNPs bound on one microsphere can be quantified using the $\Delta z$ values. In the present experiment, it was difficult to capture a single EP using an acoustic standing wave. Therefore, the behavior of the EPs as an aggregate was studied to confirm that the present concept can be used for the detection of surface reactions and has potential for trace analysis.

The avEPs were mixed with bAuNPs to obtain AuNP-coated EPs. Figure 5 shows that the levitation position of the EPs is lowered by reactions with the bAuNPs. Obviously, avEPs coated with 100-nm bAuNPs (EP-100AuNPs) are levitated at a lower position than those coated with 50-nm bAuNPs (EP-50AuNPs), as indicated by Fig. 4. The curve fitting of the $\Delta z$-$V$ curves with Eq. (3) allowed us to estimate the densities of the EP-50AuNPs and EP-100AuNPs to be 1.416 and 1.518 g cm$^{-3}$, respectively. These density changes are induced when a single EP captured 4000 50 nm-AuNPs and 1000 100 nm-AuNPs. According to the amount of moles of avidin bound on the EPs (Table 2), $1 \times 10^4$ avidin molecules are present on a single EP, on average. This indicates that only a quarter of the total avidin molecules on the EPs reacted with the 50 nm-bAuNPs, and that reacted far less with the 100 nm-bAuNPs. Four biotin molecules can bind one avidine molecule. However, when a bAuNP is associated with an avidin molecule on the EP surface, it interferes with access of other bAuNPs to nearby avidin molecules, as well as to any unoccupied sites on the same avidin molecule, owing to a steric hindrance. Because this interference is more significant for the larger 100-nm AuNP, fewer 100-nm AuNPs were bound on the avEPs. However, despite the fewer numbers of captured AuNPs, the 100-nm AuNPs yielded a larger $\Delta z$.

![Fig. 3 Schematic of the reaction between an MP and AuNPs. In the present case, the association between avidin and biotin was utilized for the binding of MPs and AuNPs. Because the resulting AuNP-coated MPs had a higher density than unreacted MPs, the levitation coordinate was shifted downward by the reaction.](image)

![Fig. 4 Relationship between the number of AuNPs bound on one avEP and (A) the density or (B) $\Delta z$ of the MP. The diameter of the EP was assumed to be 4.4 $\mu$m, with $E_{ac} = 0.1$ Jm$^{-3}$.](image)

### Table 2 Characterization of the EP microspheres

| Diameter/ $\mu$m | Density/ g cm$^{-3}$ | Weight per particle/g | Specific surface area/ cm$^2$ g$^{-1}$ | Avidin on EP/mol g$^{-1}$ |
|------------------|----------------------|-----------------------|---------------------------------------|--------------------------|
| 4.41 (2.29)$^a$  | 1.304                | $1.10 \times 10^{-7}$ | $1.38 \times 10^{4}$                | $1.43 \times 10^{-10}$  |

$^a$ Standard deviation.
The avEP microspheres reacted with different amounts of 100-nm bAuNPs, and the resulting EP-100AuNPs were studied in an acoustic field (Fig. S5, Supporting Information). The number of bAuNPs bound on an EP varied in the range of \( n = 170 - 800 \). The levitation position moved downward as more AuNPs were bound on the EPs. Figure 6 shows the relationship between \( n \) and \( \Delta z \) measured at \( V = 5.0 \) V; a linear relationship is observed up to \( n = 600 \). At \( n = 800 \), the solution remained reddish after the reaction, suggesting that not all of the bAuNPs were bound to the avEPs. During the reaction, some bAuNPs were adsorbed on the wall of a reservoir. The variations of the removed amounts of bAuNPs resulted in the large standard deviations shown in this figure. Improved repeatability is an important issue to be considered as the next step. The standard deviations range from \( \Delta z = 5 \) to \( 8 \) \( \mu m \). When \( 3\sigma \) is taken as the detection limit, \( 200 - 300 \) AuNPs per EP can be detected. Since the binding of AuNPs on an EP is mediated by avidin-biotin reaction in the present case, \( 200 - 300 \) molecules can eventually be detected using one microparticle.

Conclusion

The proposed method can be applied to other surface reactions, such as DNA matching and antigen-antibody reactions by simply changing the mediated molecules or reaction systems. Thus, the present method is highly versatile and allows for the flexible design of bioanalytical systems. Furthermore, the present study demonstrated the potential of the acoustic-gravitational field as a highly sensitive measurement tool. In the present configuration, several tens of MPs were introduced into a cell, and their behavior as an aggregate was investigated. If a single MP is examined, tens of AuNPs or mediated molecules can be detected according to the levitation coordinate of the MP. Moreover, the reaction progress can be followed using the same setup; the response of the MPs in the acoustic field is fast enough for reactions completed within several seconds to be followed. Because any solvent or aqueous media can be used in the combined acoustic-gravitational field, the present approach has wide applicability. We are studying further technical improvements of the present method so as to allow studies involving a single MP. Highly sensitive analysis enabling zepto to yocto mole detection should be possible using this approach and will be reported in the near future.

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