Enhanced Optical Collection of Micro- and Nanovesicles in the Presence of Gold Nanoparticles

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ABSTRACT: We describe a process for collecting micro- and nanovesicles on a glass substrate using the optical pressure of a laser beam. The laser beam was focused on a glass substrate that sandwiched a solution containing vesicles prepared using a phospholipid. The optical pressure generated at the surface of the vesicles pulled them into the center of the beam where they formed an aggregate on the glass surface. The vesicles prepared with a buffer solution were successfully collected via adsorption onto the glass surface, whereas the vesicles prepared with pure water exhibited no such tendency. The time required to collect a certain amount of vesicles was inversely proportional to their concentration. To enhance the collection efficiency, we added gold nanoparticles to the vesicle solution. The addition of gold nanoparticles into the solution reduced the collection time to one-tenth of that without it, and this was attributed to thermal mixing promoted by the heat generated by the absorption from the gold nanoparticles in the solution, as well as to an enhancement of light scattering induced by the gold nanoparticles. The optical collection of vesicles coupled with gold nanoparticles shows a promise for the collection of trace amounts of extracellular vesicles in biological fluids.

INTRODUCTION

Biological cells release several types of extracellular vesicles: exosomes, microvesicles, and apoptotic bodies. Among these, exosomes have attracted much attention since 2007 when Lötvall’s group discovered that micro-RNA resides in the body of exosomes. After that finding, many studies have focused on the characterization and analysis of exosomes to clarify their functions, roles, and utility. Exosomes are also expected to be novel biomarkers for cancer because they should contain information on the cells that release them.

Exosomes are found in biological fluids such as serum, saliva, and urine, although their functions are not completely understood. Clarification of the functions and characteristics of exosomes and their components will require separation, concentration, and detection techniques. Some of the well-known standard methods for collecting exosomes include ultracentrifugation and differential ultracentrifugation. Other separation methods include size exclusion chromatography and field-flow fractionation. Microfluidic devices show a promise as platforms for the isolation, collection, and detection of extracellular vesicles.

Compared with these separation methods, manipulation using optical pressure, employed generally in laser trapping, optical trapping, and optical tweezers, could be useful for the collection of small vesicles. For example, in 1995, we developed a separation method for micrometer-sized particles using optical pressure referred to as optical chromatography that permitted the separation of polymer particles and biological cells depending on their shapes and refractive indices. Optical chromatography was expanded to microfluidic systems to separate, concentrate, and fractionate polymer particles. Furthermore, Hart et al. demonstrated interesting results showing that optical chromatography can discriminate between two closely related Bacillus spores. Conversely, we recently reported the manipulation of oil droplets using optical pressure to trap, contact, and coalesce oil droplets stabilized by an emulsifier. These articles demonstrated the usefulness of optical pressure for collecting micrometer-sized particles, droplets, vesicles, and biological cells.

In this study, we employed optical pressure in the collection of micrometer- and nanometer-sized vesicles on a glass substrate in a solution. The solution containing the vesicles was sandwiched between two glass plates, and a laser beam was focused onto one of them. The laser beam accelerated the propagation of the vesicles into the direction of the beam and dragged them to its center, which resulted in an aggregation of the vesicles onto the glass plate. To enhance the collection efficiency, the vesicles were mixed with gold nanoparticles that were expected to adsorb onto the surface of the vesicles and enhance the scattering of light. The effect of the gold nanoparticles was evaluated according to the time required to collect a certain amount of the vesicles on the glass substrate.

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RESULTS AND DISCUSSION

Collection of Vesicles. In this study, micro-and nanovesicles were collected on a glass substrate via optical pressure. In a preliminary study, we prepared vesicles with a diameter of 1.0 μm using deionized water and attempted to collect them on a hydrophilic glass substrate. The collection of the vesicles prepared with deionized water is shown in Figure 1a,b. When the laser irradiated a suspension of vesicles, the vesicles were accelerated to the propagation direction of the laser beam via a scattering force and were dragged into the center of the beam by its gradient force, which is similar to the process of optical chromatography.14 An aggregate of the vesicles was formed at the beam spot of the laser on the glass substrate with time (Figure 1a). When the laser was turned off, however, the vesicles were immediately dispersed (Figure 1b). These results indicate that the vesicles were collected on the glass surface via the optical pressure, but they were not adsorbed onto the glass surface.

Adsorption of the vesicles would be related to their surface charge, which depends on the conditions of the surrounding medium. According to the literature,23 the electric charges of phospholipids vary depending on the pH and coexisting ions in the medium. Phosphatidylcholine, for example, is positively charged in an acidic pH lower than 3 and then suddenly is negatively charged in water without salts. Conversely, in the presence of NaCl, the charge of the vesicles changes gradually and becomes almost neutral in a pH ranging from 3 to 7. Therefore, in deionized water, the vesicles would be negatively charged, resulting in no adsorption on the surface of the hydrophilic substrate because of the electrostatic repulsion.

To fix the vesicles on the glass substrate after turning off the laser, we employed phosphate-buffered saline (PBS) instead of deionized water to hydrate dipalmityloyphtalophosphatidylcholine (DPPC). The collection of the vesicles prepared with PBS is shown in Figure 1c,d. The vesicles prepared with PBS were successfully adsorbed onto the glass surface, even when the laser was turned off (Figure 1d). The increased size of the aggregate can be seen over time. It is obvious that the surrounding medium must contain salts to adsorb the vesicles onto a glass substrate.

The collection speed was decreased with decreases in the concentration of DPPC that corresponded to the number of vesicles. The time required to collect a constant amount of vesicles was inversely proportional to the concentration of the DPPC. The time also increased when the size of the vesicles was reduced to 100 nm, even though the required number of vesicles was greater than 1.0 μm. For example, the times required to collect an aggregate of 11 μm were 3 min for 1.0 μm vesicles and 25 min for 100 nm vesicles at 0.25 mM DPPC. This fact can be explained by the dependence of the optical pressure on the size of the particle because the intensity of the light on the surface of a particle increases with increases in the size of the particle. Therefore, the collection speed will definitely increase with increases in the power of the laser because the optical pressure is proportional to the power of the laser.

Enhancement of Collection Speed with Gold Nanoparticles. To evaluate the collection speed, we determined the time required to form 11 μm of an aggregate on a glass substrate at different concentrations of DPPC, as shown in Figure 2 (red circle). The times totaled to 4 min at 0.25 mM DPPC and more than 100 min at 0.01 mM DPPC, respectively. Although a time of 100 min is much shorter than what is required for ultracentrifugation that last for more than a day, further improvement in the collection efficiency is preferable for practical applications. Therefore, we attempted to employ gold nanoparticles because these were expected to enhance the light scattering of the vesicles via adsorption onto the surfaces of the vesicles.

We added two different gold nanoparticles, 4-dimethylaminoptyridine (DMAP)-coated and citrate-coated, to a suspension of the vesicles. These nanoparticles were expected to exhibit different behaviors during adsorption onto the vesicles because DMAP and citrate ions are cationic and anionic, respectively. Therefore, these gold nanoparticles were mixed with 1.0 μm suspension of vesicles.

The effect of citrate-coated nanoparticles is shown in Figure 2 (blue circle). The gold nanoparticles shortened the collection time to one-tenth at 0.01 mM DPPC. We speculated that DMAP-coated gold nanoparticles would be adsorbed onto the vesicles more efficiently than citrate-coated versions because the vesicles would theoretically have a weak negative charge. However, no difference was observed between DMAP-coated
and citrate-coated gold nanoparticles. This fact implies that both anionic and cationic gold nanoparticles would be adsorbed onto vesicles in the same degree because they have a neutral surface charge. Otherwise, the adsorption of gold nanoparticles would not be essential for enhancing the collection efficiency.

It is interesting that under laser irradiation, the behaviors of the vesicles appeared different in the presence of gold nanoparticles. In the presence of the gold nanoparticles, the vesicles were vigorously stirred, as shown in the Supporting Information videos (Video S1 with and S2 without gold nanoparticles). The difference could have been because of thermal convection induced by increases in temperature via the light absorbed by the surrounding gold nanoparticles. The uncaptured vesicles seemed to disperse radially from the beam spot in the presence of gold nanoparticles (Video S1), whereas they moved randomly in the absence of the gold nanoparticles (Video S2). The radial dispersion may have been caused by the increased temperature within the laser beam when the solution, heated by the light, generated convection to the outside of the beam. This phenomenon was observed for both DMAP-coated and citrate-coated gold nanoparticles, independent of the stabilizing agent. This could be attributed to the similar molar extinction coefficients of these gold nanoparticles at 532 nm (3.4 × 10^7 L mol^-1 cm^-1 for citrate-coated gold nanoparticles and 2.1 × 10^9 L mol^-1 cm^-1 for DMAP-coated gold nanoparticles).

On the basis of these results, the enhanced collection efficiency in the presence of gold nanoparticles could be explained mainly by thermal convection of the vesicles via light absorption by the free gold nanoparticles. Other possible factors include collisions of accelerated gold nanoparticles with vesicles and an enhancement in the light scattering of the vesicles induced by the gold nanoparticles adsorbed onto the vesicles. The thermal convection increased the probability that the vesicles passed transversely across the laser beam. The gold nanoparticles adsorbed onto the vesicles would generate strong optical pressure via enhanced scattering originating from the surface plasmon. The free gold nanoparticles also accelerated in the propagation direction of the laser beam; therefore, they may have accelerated the vesicles when they collided with them.

We needed to recover the vesicles collected on the glass surface for subsequent analysis. However, the adsorption of the vesicles was too weak for recovery, so that they were peeled off the glass substrate when it was removed from the well. Therefore, further improvements in the collection system are required to apply the collected vesicles to further chemical analysis. Examples of these improvements could include either the use of a microfluidic device or a flow system coupled with an analytical instrument.

**CONCLUSIONS**

We have developed a method to collect vesicles on a glass substrate via the optical pressure of a laser beam. The surrounding medium must contain salts to control the surface charge of the vesicles. A medium of biological fluids, in general, will contain an amount of salt sufficient to allow the analysis. We also found that the addition of gold nanoparticles significantly improved the collection efficiency. The effect of the gold nanoparticles was attributed to thermal convection in the vesicles via light absorption by the free gold nanoparticles and by collisions of the accelerated gold nanoparticles with the vesicles, which enhanced the light scattered among the vesicles. Further investigation is necessary, however, to clarify the mechanisms of the enhanced collection efficiency. The use of gold nanoparticles in optical manipulation would be useful for efficiently collecting micro- and nanovesicles in small volumes of biological fluids.

**EXPERIMENTAL SECTION**

**Materials.** All of the reagents used in this study were of analytical grade. Deionized water was prepared by means of an Elix water purification system (Millipore Co. Ltd., Molsheim, France). DPPC was obtained from Avanti Polar Lipids (Alabaster, AL, USA). PBS was purchased from Thermo Fisher Scientific (Yokohama, Japan). Acetone, chloroform, tolulene, sodium sulfate anhydrous (Na2SO4), sodium borohydride (NaBH4), sodium tetrachloroaurate(III) dihydrate (NaAuCl4·2H2O), DMAP, and tetraoctylammonium bromide were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ethanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sulfuric acid (H2SO4), hydrochloric acid, and 30% hydrogen peroxide (H2O2) were obtained from Kanto Chemical (Tokyo, Japan).

**Preparation of Vesicles.** A 4.15 μL aliquot of 30 mM DPPC in chloroform was transferred to a glass tube. The chloroform was evaporated via injection of nitrogen gas. After the removal of chloroform, the glass tube was placed in a vacuum desiccator for 1–3 h. To prepare the vesicles, 500 μL of deionized water or PBS was added to the glass tube followed by further sonication (55 °C for 30 s) and stirring (for 30 s) thrice. The size of the vesicles was adjusted using a Mini-Extruder (Avanti Polar Lipids) with a 1.00 or 0.1 μm polycarbonate membrane. The final concentration of DPPC in the suspension was estimated at 0.25 mM. The suspension was successively diluted to prepare suspensions with concentrations lower than 0.25 mM.

**Preparation of Gold Nanoparticles Coated with DMAP.** We prepared gold nanoparticles coated with DMAP according to a procedure found in the literature.26 A 20 mL aliquot of 100 mM tetracylammonium bromide dissolved in toluene was gathered in a separation funnel. A 7.5 mL aliquot of 20 mM NaAuCl4 aqueous solution was added to the separation funnel, followed by shaking to mix the organic and aqueous phases. The solution was set to stand, and then the metal salt was transferred to the toluene phase within a few seconds. Then, 6.25 mL of freshly prepared 0.4 M NaBH4 was added to the mixture, which caused an immediate reduction of the Au(III). After 30 min, the aqueous phase was removed, and the toluene phase was washed with 10 mL of 0.1 H2SO4, 10 mL of 0.1 M NaOH, and 10 mL of deionized water thrice, followed by drying with anhydrous Na2SO4. An aqueous solution of 1 mM DMAP (1 mL) was added to 1 mL of the toluene phase, and the mixture was shaken, which resulted in DMAP-coated gold nanoparticles in the aqueous solution. The size of the nanoparticles was estimated at 25 nm based on the UV–vis spectrum.27

**Preparation of Gold Nanoparticles Coated with Citrate.** Citrate-coated gold nanoparticles were prepared according to a procedure cited in the literature.27 A 100 mL aliquot of deionized water was placed in a sample bottle, followed by the addition of 500 μL of 0.059 M NaAuCl4 and 3.5 mL of 3% (w/v) trisodium citrate solution. The mixture was heated at 80 °C for 3 h to reduce Au(III) to Au(0). The reaction was quenched by cooling to room temperature, which changed the solution color from yellow to red. The sizes of the
nanoparticles were estimated at 30 nm based on the UV-vis spectrum.27

**Preparation of Samples.** We prepared three different samples containing vesicles: (i) a mixture of a vesicle suspension and PBS at a ratio of 1:1; (ii) a mixture of a vesicle suspension and 0.3 nM DMAP-coated gold nanoparticle solution at a ratio of 1:1; and (iii) a mixture of a vesicle suspension and 0.3 nM citrate-coated gold nanoparticle solution at a ratio of 1:1. These mixtures were directly introduced into a well for collecting the vesicles via optical pressure.

**Fabrication of the Collection Well.** Piranha treatment was performed to hydrophilize the glass surface by immersing the glass substrate overnight into a mixture of H2SO4 and 30% H2O2 at a ratio of 1:3. The hydrophilic glass substrate was washed with deionized water and dried at room temperature.

A collection well was fabricated by sandwiching a silicon rubber sheet (thickness, 1 mm, AS ONE, Osaka, Japan) between two glass substrates [glass slide (thickness, 1.0 mm) or cover glass (thickness, 0.12–0.17 mm)]. The silicon rubber sheet was cut in a square shape (18 × 18 mm) with a 5 mm diameter hole at the center. The silicon rubber sheet was placed on a glass slide, and then a sample solution was poured into the hole. The hole was covered with either a cover glass or a glass slide that was firmly attached to the silicon rubber sheet to prevent leakage of the solution.

**Experimental Setup.** The experimental setup is shown in Figure 3. An Nd:YAG laser (532 nm, maximum power, 3 W, focused onto the surface of the upper glass substrate. The hole was covered with either a cover glass or a glass slide that was firmly attached to the silicon rubber sheet to prevent leakage of the solution.

**Video S1, Collection of vesicles in the presence of gold nanoparticles (MPG)**

**Video S2, Collection of vesicles in the absence of gold nanoparticles (MPG)**

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**Notes**

The authors declare no competing financial interest.

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