Hollow Silica Microspheres for Buoyancy-Assisted Separation of Infectious Pathogens from Stool

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ABSTRACT

Separation of cells and microorganisms from complex biological mixtures is a critical first step in many analytical applications ranging from clinical diagnostics to environmental monitoring for food and waterborne contaminants. Yet, existing techniques for cell separation are plagued by high reagent and/or instrumentation costs that limit their use in many remote or resource-poor settings, such as field clinics or developing countries. We developed an innovative approach to isolate infectious pathogens from biological fluids using buoyant hollow silica microspheres that function as “molecular buoys” for affinity-based target capture and separation by floatation. In this process, antibody functionalized glass microspheres are mixed with a complex biological sample, such as stool. When mixing is stopped, the target-bound, low-density microspheres float to the air/liquid surface, which simultaneously isolates and concentrates the target analytes from the sample matrix. The microspheres are highly tunable in terms of size, density, and surface functionality for targeting diverse analytes with separation times of ≤ 2 minutes in viscous solutions. We have applied the molecular buoy technique for isolation of a protozoan parasite that causes diarrheal illness, *Cryptosporidium*, directly from stool with separation efficiencies over 90% and low non-specific binding. This low-cost method for phenotypic cell/pathogen separation from complex mixtures is expected to have widespread use in clinical diagnostics as well as basic research.

Keywords: Bioseparation, sample preparation, analyte concentration and isolation, buoyancy cell separation, glass microbubbles

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1. INTRODUCTION

Separation of disease-related cells and microorganisms from complex biological mixtures is a critical first step in many analytical applications ranging from clinical diagnostics to environmental monitoring for food and waterborne pathogens [1]. Existing separation methods can be loosely grouped into two categories; those that rely upon physical differences among cells (i.e. size, shape, density, or electrophoretic mobility); and those that rely upon differences in chemical composition (i.e. protein or other cell surface markers) [1, 2]. The most common physical separation methods include size-dependent micro/ultrafiltration and density-based centrifugation using Percoll[®] or Ficoll-Paque[®] density gradients [1, 3]. These methods typically have the advantage of being label-free and non-destructive which allows for subsequent cell culture or growth; however, they depend upon a large physical difference between target and non-target materials, limiting the ability to discriminate between closely-related populations [2]. Affinity-based techniques, such as magnetic-activated cell separation (MACS) and fluorescence-activated cell separation (FACS) overcome these limitations through the use of target-specific antibodies to impart higher selectivity for the particular cell type or pathogen of interest [2, 4, 5]; however, high reagent and/or instrumentation costs continue to limit their use in many remote or resource-poor settings. Even microfluidic and lab-on-a-chip platforms for cell separation remain limited in their widespread clinical adoption due to complexity and cost.

Recently, a few studies have emerged describing a buoyancy-activated cell sorting (BACS) technique that uses low-density hollow glass microbubbles for separation of CD4⁺ T-cells and circulating tumor cells from whole blood, and even cancer stem cells from heterogeneous cell populations [6-8]. Advantages of BACS include (i) low material cost of the glass microbubbles, which are an industrial additive used in plastics and coatings available in

kilogram quantities, and (ii) the simplicity of the floatation-based separation, which eliminates the need for any external instrumentation like a centrifuge or magnet. In addition, surface functionalization of glass microbubbles can take advantage of the well-established silica-based chemistries for direct conjugation of proteins, antibodies, or other affinity ligand [9].

In the current study, we describe for the first time a BACS process for the isolation of an infectious disease pathogen from perhaps the most complicated biological sample type, stool. A schematic of this “molecular buoy” method for isolation of *Cryptosporidium*, a protozoan parasite that causes diarrheal illness, is illustrated in Fig 1. Here, a complex biological sample is incubated with functionalized hollow microspheres and mixed in order to facilitate target capture of the analytes. When mixing is stopped, the target-bound low-density microspheres migrate to the air/liquid interface where they float at the surface, which simultaneously isolates and concentrates the target analytes from the sample matrix.

We chose *Cryptosporidium* as our initial target pathogen since passive fecal floatation is a technique that has long been used for the recovery of parasitic eggs and oocysts in stool prior to microscopic examination and diagnosis in humans and pets [10-12]. The conventional stool floatation method relies on the differences in the specific gravity of the eggs/oocysts, fecal debris, and the floatation solution [11]. Some common floatation solutions include modified Sheather’s solution, zinc sulfate and magnesium sulfate with a specific gravity in the range of 1.18-1.27 (specific gravity of water is 1.0) [11]. Parasitic eggs/oocysts that have a lower specific gravity (between 1.05-1.15) will, over time or under centrifugal force, float to the surface while heavier debris will sink to the bottom [11]. Drawbacks of this technique include low recovery rates, the remaining need for centrifugation, and the constraint to organisms that have an inherently low specific gravity, such as eggs and encapsulated cysts, which can have gas or air

pockets that lower their overall density. Using the molecular buoy approach, we aim to overcome these restrictions and generate a high affinity, density-based separation technique that uses low-cost materials, eliminates any external instrumentation or centrifugation, and can be universally applied to isolate a variety of clinical pathogens and biomarkers.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

We obtained the H20 and H50 hollow silica microspheres, with an epoxy silane surface treatment and a density of 0.2 g/cm³ and 0.5 g/cm³, respectively from 3M (H20/H50 glass bubbles; 3M, St. Paul, MN). Buffers, glycerol, protein G, and L-cysteine were all purchased from Thermo Fisher Scientific (Waltham, MA). Phosphate buffered saline (PBS) was prepared in deionized water using BupH™ Modified Dulbecco's PBS (#28374) buffer packs with a final concentration of 8 mM sodium phosphate, 2 mM potassium phosphate, 0.14M sodium chloride and 10 mM potassium chloride at a pH of 7.4. *C. parvum* oosysts (Iowa isolate, #P102C) and negative control stool were purchased from Waterborne Inc. (New Orleans, LA). Antibodies included an unconjugated anti-*C. parvum* monoclonal mouse IgG3 (clone BEL 0126, #64526, Novus Biologicals, Littleton, CO), an anti-*C. parvum* directly conjugated to Alexa-Fluor®488 (clone BEL 0126, #2402-3007AF488, Bio-Rad AbD Serotec, Raleigh, NC) and a goat-anti-mouse (GAM) IgG conjugated to Alexa-Fluor®488 (#A11001, Invitrogen, Carlsbad, CA).

2.2 Microsphere Size and Floatation Kinetics

The H20/H50 glass microspheres were suspended in PBS at a final concentration of 1.0 mg/ml. A wet-mount slide using ~10 µl of the microsphere solution was prepared for imaging on an EVOS FL Auto Cell Imaging System (Thermo Scientific, Waltham, MA) using an Olympus

UPlanApo 10X objective (0.4 NA). The diameter of at least 5,000 microspheres was measured directly from transmitted light microscopy images using ImageJ open-source software [13]. ImageJ particle analysis criteria applied to binary, thresholded images included a minimum circularity of 0.85 in order to eliminate non-solitary or broken microspheres from the size analysis. Histograms with a bin size 4 μm were prepared in SigmaPlot 12.5 (SyStat Software, Inc., San Jose, CA), along with all other graphs.

Additional size fractionation of the H2O microspheres was achieved using a stacked set of wire mesh test sieves (VWR, Radnor, PA) with standard mesh sizes of 140, 200, 270, and 500 that correspond to a nominal opening of 105 μm , 74 μm , 53 μm , and 25 μm , respectively. In this process, 1 g of dry H2O microspheres were placed in the upper sieve and shaken overnight (>12 hrs) on a rotary shaker at 350 rpm. Each fractionated population was decanted from the respective sieve and stored at room temperature in clean glass vials.

The floatation and buoyancy properties of the silica microspheres were characterized using a UV/Vis spectrophotometer (Cary 60 UV/Vis; Agilent Technologies, Santa Clara, CA) to monitor the change in optical density (OD) at 450 nm over 10 min as the particles floated to the surface of a 2 ml quartz cuvette. Separation time was calculated from the kinetic data as the time point at which 90% of the total OD decline occurred ($0.9 \times (\text{OD}_{\text{initial}} - \text{OD}_{\text{final}})$). The various glycerol concentrations, from 0% – 50% in PBS, were used in this characterization to simulate the wide range of fluid densities and viscosities expected in biological fluids.

2.3 Surface Functionalization

The epoxy silane reactive groups were used to directly conjugate protein G to the microsphere surface via a pH-dependent ring-opening reaction [9]. Here, protein G (0.01 mg/ml – 1.0 mg/ml) was incubated with 1 mg of the 25-53 μm fractionated microspheres in 100 μl (1.4

× 10⁶ microspheres/ml) of PBS buffer (pH 7.4) or 0.1 M sodium carbonate/bicarbonate buffer at various pH (range 9.1 – 10.5) [14] for 24 hrs at 4°C with continuous end-over-end mixing. L-cysteine was added to a final concentration of 50 mM and incubated for another 24 hrs in order to block any unreacted epoxy groups. Next, we washed the microspheres three times in PBS by tilting the microfuge tube ~45° allowing the particles to collect at the liquid surface, then removing the lower solute fraction and resuspending remaining particles in 100 – 200 µl buffer. Functionalized particles were typically used immediately or stored at 4°C for up to one week.

The presence of protein G on the microsphere surface was confirmed indirectly by fluorescence microscopy and quantitative image analysis of protein G bound to a secondary GAM/Alexa-Fluor[®] 488 antibody. GAM binding was carried out at a final concentration of 0.1 mg/ml in PBS, mixed with 0.1 mg protein G-functionalized microspheres for 2 hrs at room temperature. Epi-fluorescent images were collected from at least 10 fields-of-view and exported to ImageJ for quantitative analysis. Analysis routines included automatic intensity thresholding, conversion to a binary mask, holes filled, and watershed applied to obtain region-of-interest (ROI) outlines for each particle with a minimum size of 500 pixels and circularity of 0.75. The intensities within the ROIs were measured on the original 8-bit grayscale image to obtain mean fluorescence intensity (MFI) per microsphere. Approximately 2,000 particles were measured at each protein G concentration run in triplicate. Data was graphed in SigmaPlot and fit to an exponential rise-to-maximum curve with equation: $f = y_0 + a(1 - e^{-bx})$. Microsphere concentrations (beads/ml and beads/mg) were obtained from hemocytometer counts of 1 mg of beads from the 25 – 53 µm fraction resuspended in 100 µl 50% glycerol/PBS.

2.4 Capture of *Cryptosporidium parvum* oocysts

Protein G-coated microspheres were prepared as described above using 0.1 mg/ml protein G in 100 μ l sodium carbonate/bicarbonate buffer at pH $\leq$ 9.4. Blank control microspheres did not contain protein G (i.e. buffer only). For capture studies, protein G-coated and control microspheres were incubated with unconjugated anti-*Cryptosporidium* IgG (0.01 mg/ml in PBS) for 2 hrs at room temperature and washed three times by floatation. Microspheres were then mixed with either buffer or stool samples spiked with 10^2 - 10^5 oocysts in 100 μ l for up to 2 hrs with continuous end-over-end mixing. Following a 1-2 min floatation-based separation step, the lower fraction was transferred to a new microfuge tube where any unbound oocysts were counted using a hemocytometer. At the lower oocyst concentrations, centrifugation and resuspension of the recovered fraction (5,000 rpm for 5 min, supernatant removed and pellet resuspended in 20 μ l) was needed for counting. The difference between the total number of oocysts input into each sample and the number recovered in the un-bound fraction was used to calculate the percent oocysts bound by the functionalized molecular buoys. Three independent replicates at each oocyst concentration were run in buffer and two in stool. A one-tailed t-test was performed in SigmaPlot to determine significant differences between positive/negative control samples and between PBS/stool samples containing the same number of oocysts ($p < 0.05$). Negative control stool was purchased from a commercial vendor, Waterborne Inc. (New Orleans, LA) and consisted of roughly 10% insoluble solid material and 90% water. Since *Cryptosporidium* infection causes diarrhea, characterized by loose or watery stool with a water content $>80\%$ [15], the stool sample used in this study closely mimicked this condition without further dilution or solubilization.

For qualitative imaging studies, we replaced the unconjugated antibody with an Alexa-Fluor[®]488 conjugate of the same monoclonal antibody clone at 0.1 mg/ml in PBS. In addition, the *Cryptosporidium* oocysts were added immediately after the 2 hr antibody incubation, without washing excess antibody from the functionalized microspheres. Thus, capture and immunolabeling of oocysts was allowed to occur simultaneously and enabled visualization of both the microspheres and bound *Cryptosporidium* via fluorescence. Confocal imaging was performed on an Olympus FV100 Scanning Confocal Microscope with a 60X PlanApoN oil-immersion objective (1.4 NA) (Olympus Corporation, Tokyo, Japan). Alternatively, we pre-labeled and washed the oocysts (0.01 mg/ml anti-Crypto/Alexa-Fluor[®]488 for 2 hrs followed by centrifugation at 5,000 rpm, supernatant removed) then added them to protein G-functionalized microspheres for capture of the antibody:oocyst immunocomplexes, followed by epifluorescence imaging as previously described.

3. RESULTS AND DISCUSSION

3.1 Characterization of size and buoyancy properties

We measured the diameter of H20 and H50 microspheres from microscopy images and generated histogram plots to examine the distribution of bead sizes present in each population. We found a wide range of particle sizes in the H20 population, from less than 10 μm in diameter to more than 80 μm in diameter, with a population mean of $27 \pm 13 \mu\text{m}$ and a mode of 16 μm (Fig 2A). The H50 microspheres were slightly smaller with a mean diameter of $17 \pm 7 \mu\text{m}$ and a mode of 12 μm (Fig 2B).

Next, we monitored the rate of microsphere separation by time-lapse video and optical spectroscopy (Fig 3). In PBS buffer, the H20 microspheres immediately began to migrate

upward when mixing was stopped and, after roughly 2 min, nearly all of the microspheres could be seen floating at the surface with cleared lower fluid fraction (Fig 3A; full video available in Supplemental Material). Kinetic traces monitoring the change in optical density throughout this floatation-based separation process in PBS buffer, and solutions containing increasing amounts of glycerol, are shown in Fig 3B and 3C. The fastest decline in OD was detected in PBS buffer, or 0% glycerol, indicating the highest separation rate in this solution with low density, low viscosity. As glycerol content increased up to 50%, the rate of OD decline was progressively delayed for both the H20 and H50 glass bubbles. Using the kinetic data, we calculated the experimental separation time as the time point at which 90% of the total OD change had already occurred. For the H20 glass microspheres, the separation time was 2.7 min in PBS buffer and 6.7 min in the 50% glycerol/PBS solution, while the slightly smaller H50 glass microspheres had a separation time of 4.7 min in buffer and 8.2 min in 50% glycerol. A plot H20 separation time versus fluid viscosity closely followed the non-linear relationship between glycerol content and viscosity (Fig 3B, inset), suggesting that viscosity is a key parameter influencing separation time and efficiency. A summary of all experimentally derived separation times is provided in Supplemental Table 1, along with published values for fluid viscosity and density of the glycerol solutions used [16, 17].

3.1.1 Theoretical considerations

These results can be better understood by revisiting a few principles of physics which describe the three forces acting on the particles (Fig 4). For a submerged object, the upward buoyant force is equal to the weight of the displaced fluid (Archimedes' principle) and can be calculated according to Equation 1 where ρ_f is the density of the fluid, V_f is the volume of the displaced fluid, and g is gravity (9.8 m/s^2). Submerged objects that rise in a fluid have a buoyant

force that is greater than the sum of the two downward forces due to gravity and drag. The force of gravity is simply the weight of the object (mg), and can be written according to Equation 2 where ρ_o is the density of the object, V_o is the volume of the object, and g is gravity. The drag force, also known as the frictional force or Stokes' drag, on the object is given by Equation 3 where η is the fluid viscosity, r is the radius of a spherical object, and v is the velocity [18].

When the net force is positive ($F_{buoyancy} > F_{grav} + F_{drag}$), the object will accelerate upwards until the object reaches equilibrium where the net force is zero (the sum of forces $F_{net} = 0$) and the object is rising at a constant speed or it is floating on the surface of the fluid. Since the silica microspheres are hollow, the buoyant force is largely size-dependent with larger particles having less density and displacing a larger volume of fluid than smaller microspheres in a fluid with similar density/viscosity. As such, the magnitude of the positive net force is greater, despite an increase in both the gravity and drag force for larger particles than smaller particles. For example, the buoyant force acting upon the H20 microspheres with a mass of 2.06 ng is 1.01×10^{-7} N (calculated from Equation 1 and mean volume of the microspheres) while the H50 microspheres with a mass of 1.29 ng experiences a buoyant force of 2.52×10^{-8} N such that there is only 1.6-fold difference in mass, but 4-fold difference in the buoyant force. Thus, the larger and less dense hollow microspheres would be expected to have faster separation times in fluids with similar density/viscosity properties, as seen in the kinetic data above comparing separation times between the larger H20 (2.7 min in 0% glycerol) and smaller H50 microspheres (4.7 min in 0% glycerol). In addition, for similarly sized microspheres in high viscosity solutions, an increase in the drag force would be expected to reduce the net force and delay separation time, as seen when the H20 separation time was plotted against viscosity (Fig 3). Thus, for application development, this relationship was critical for selection of an appropriately sized microsphere

population in order to achieve a desired separation time in a given biological matrix of known viscosity.

3.1.2 Size fractionation of H2O microspheres

Because the buoyant force was largely size-dependent in fluids with similar density/viscosity properties, we size fractionated the bulk H2O microspheres in order to obtain a more homogeneous population which would enable more precise control over the buoyancy properties and ultimately, the separation parameters in viscous fluids. Using wire mesh test sieves with nominal mesh sizes of 105 μm , 74 μm , 53 μm , and 25 μm , respectively, we obtained four relatively homogeneous bead populations with mean diameters ranked from smallest to largest of $21 \pm 5 \mu\text{m}$, $38 \pm 7 \mu\text{m}$, $62 \pm 10 \mu\text{m}$, and $81 \pm 12 \mu\text{m}$ (Fig 5A, panels i – iv). Histogram plots of each population are shown in Fig S1. An insufficient number of microspheres were larger than the 105 μm sieve size for measurement of the mean diameter in that population. The vast majority of the microspheres were found in the $< 25 \mu\text{m}$ dia. fraction and a few of these smaller particles could still be seen in the larger sieved populations due to imperfect sifting or attachment to larger microspheres.

We again monitored the rate of microsphere separation for each of these size-fractionated populations in PBS and 30% glycerol as previously done for the bulk microspheres. The viscosity of glycerol at 30% v/v (2.5 cP) is roughly equivalent to the viscosity of normal whole blood (3.2 cP) and plasma (2.0 cP) [19]; therefore, it was used as a surrogate for the viscosity expected in a biological fluid. As expected, separation time was inversely proportional to microsphere size with the largest particles exhibiting the fastest separation times. In PBS buffer, the three largest bead populations all exhibited separation times under 30 sec, while the separation time for the smallest ($< 25 \mu\text{m}$ dia.) population was just under 2.5 min (Fig 5B and

Table 1). In 30% glycerol, these times increased to ≤ 1 min for the two largest populations and 2.2 min for the 25-53 μm dia. population, while the smallest set at < 25 μm did not achieve measurable separation over the 10-min measurement period (Fig 5C). Again, these fractionated populations exhibited the same size and viscosity relationships to separation time as the bulk populations, but with sharper, more exaggerated changes in OD that returned to near baseline levels over shorter periods of time due to the narrower distribution of sizes present in each fractionated population. Balancing the need for rapid separation versus the importance of efficient interaction with pathogens in biological solutions, we selected the ~ 38 μm dia. population obtained from the 25 – 53 μm sieve range for additional functionalization and pathogen binding studies.

3.2 Functionalization with protein G and immunoglobulins

Our functionalization strategy relied upon a pH-dependent epoxy ring-opening reaction that covalently linked the epoxy silane treated microspheres to biomolecular functional groups (-NH₂, -SH, and -OH) in protein G (Fig 6A) [9]. Protein G is an immunoglobulin (IgG)-binding protein expressed in group C and G *Streptococcal* bacteria [20]. Since protein G binds only to the Fc region of an IgG, it was used as an orientation-specific linker to ensure that the antigen binding domains of the target-specific antibody were freely available to bind pathogen targets. Optimal conditions for protein G attachment were evaluated using a secondary GAM/Alexa-Fluor®488 antibody and quantitative image analysis under various buffer conditions. A 0.1 M carbonate/bicarbonate buffer at pH 9.0 provided the most intense and uniform coating of protein G on the microspheres (Supplemental Fig S2); although, conjugation was apparent for all buffer and pH conditions tested, presumably from attachment at multiple diverse functional groups. A protein G dilution series at steady GAM/Alexa-Fluor®488 antibody concentration exhibited a

progressive increase in fluorescence intensity with increasing amounts of protein G, up to 0.5 mg/ml (Fig 6B). Quantitative image analysis supported this dose-dependent increase, which approached 95% maximum saturation at 0.31 mg/ml of protein G when fit to an exponential curve (Fig 6C) ($R^2 = 0.94$). While this was an indirect method for measurement of protein G attachment, the low fluorescence intensity found in the negative control (no protein G) suggested that the fluorescent antibody was not interacting with the silica surface nonspecifically, but binding specifically to protein G, as was confirmed in later functional studies of antigen capture without the protein G linker. We did not choose a more direct method of quantitation using fluorescently labeled protein G because fluorophore conjugation would have utilized some or even all of the same functional groups (i.e. $-NH_2$) needed for reaction with the epoxy silane treated microspheres.

3.3 Capture and isolation of *C. parvum* oocysts in buffer and stool

Using *Cryptosporidium* as a model pathogen/analyte, we found between 88 – 97% of the *Cryptosporidium* oocysts were successfully captured and separated using the molecular buoys across a wide range of clinically relevant oocyst concentrations, from 10^3 – 10^6 oocysts/ml (Fig 7A) in buffer and stool. For example, in the presence of 10^5 oocysts per 100 μ l sample, the functionalized microspheres captured $95.1 \pm 1.9\%$ oocysts in PBS buffer and $90.4 \pm 1.6\%$ oocysts in stool versus the control microspheres without protein G, which captured less than 2% and 15% of the oocysts in PBS and stool, respectively. These differences between positive/negative samples containing 10^5 oocysts were statistically significant with p values of 0.001 for PBS and 0.007 for stool. Among the negative controls, there appeared to be a slight increase in non-specific background binding in stool versus buffer possibly due to higher protein content and interaction with reactive L-cysteine thiols; however, these differences were not

statistically significant ($p = 0.26$). Furthermore, there were no significant differences in percent oocysts captured by the functionalized microspheres in stool versus PBS at any of the concentrations tested except in the highest 10^5 samples, where the number of oocysts captured in stool was slightly lower than buffer ($p = 0.03$). It is important to note that the stool matrix used in this study was “watery” and meant to estimate the w/v content (≤ 1 mg/ml) that would be indicative of diarrheal illness symptoms, as such, non-specific background levels may be underestimated compared to clinical stool samples with a highly variable solid material content. Further optimization of surface functionalization and blocking conditions may be needed for use with patient-derived stool samples. Other limitations of this capture efficiency study include the use of an indirect method to calculate the number of oocysts bound to the microspheres by subtracting the number of un-bound oocysts counted after isolation/separation from the total number input into the spiked sample ($\text{oocysts}_{\text{bound}} = \text{oocysts}_{\text{total}} - \text{oocysts}_{\text{unbound}}$). With patient-derived stool samples containing an unknown number of oocysts, target release from the microspheres will be critical for downstream detection and/or quantitation using flow cytometry, microscopy or other technique. Methods for release may include raising or lowering the pH or adjusting the ionic strength to disrupt the antibody-antigen interaction. However, incomplete target release from the microspheres and subsequent oocyst staining/washing steps could result in oocyst loss and underestimate of the number of oocysts that were bound to the microspheres, which is why we chose the indirect method to measure capture efficiency in the current study.

We used confocal imaging to further confirm successful *Cryptosporidium* oocyst capture and separation in pure buffer solutions. Interestingly, many of the captured oocysts were found sandwiched between two of the hollow silica microspheres forming a multi-bead complex or pathogen-linked network, as demonstrated in Fig 7B. Here, two fluorescent oocysts, spherical in

shape and having a diameter of 4-6 μm each, were clearly seen bound to one or more microsphere surfaces. Additional epi-fluorescence imaging using pre-labeled *Cryptosporidium* oocysts, confirmed pathogen capture with target-specific antibodies bound to molecular buoys coated with protein G, but not to the blank control microspheres lacking protein G, where oocysts were only seen free-floating in solution (Fig S3).

These capture efficiency and microscopy results also suggest that the multiple protein-protein interactions between protein G/antibody/target were all maintained throughout the separation process and were not disrupted by frictional drag or shear forces created as the particles moved through either the buffer or stool solution. A recent publication by Hsu *et al.* described the linear relationship between the diameter of a hollow microbubble and the maximum shear stress exerted upon the target-bound particle during buoyancy-assisted cell separation [8]. They found that despite an increase in force with larger particles, the magnitude of this force was well below $1/100^{\text{th}}$ of the interaction force between the microbubble-target complex [8]. Thus, even with the relatively large microspheres ($\sim 38 \mu\text{m}$ in diameter) used in our study, compared to the size of the target pathogen (4-6 μm in diameter) we should be well below the rupture force that would cause unwanted protein-protein or target dissociation. Even when we used the largest 76-105 μm fractionated microsphere population, we found similar capture efficiency for *Cryptosporidium* oocysts ($\leq 90\%$) in PBS buffer (data not shown).

4. CONCLUSIONS

In this study we have demonstrated kinetic characterization, functionalization, and application of hollow silica microspheres (i.e. molecular buoys) that perform buoyancy-assisted cell separation for capture and isolation of an infectious pathogen from a complex biological

matrix. Separations were completed in less than 5 min with high capture efficiencies, around 90% or more. It is expected that this floatation-based process for rapid bioseparation will be applicable to diverse protein analytes, biomarkers, and pathogens for downstream disease detection and bioanalysis. Multi-plex potential exists with the use of multiple target-specific antibodies functionalized on the microsphere surface, or with the use of different sized microspheres each containing a unique target-specific antibody. Future work with *Cryptosporidium* will be focused upon optimizing target-microsphere incubation times, comparison against traditional methods such as magnetic affinity isolation, and establishing efficacy using clinical stool samples from diarrhea patients. In addition, methods for target release and downstream integration with low-cost rapid diagnostic platforms, such as paper-based microfluidic devices, are currently underway.

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Fig 1. Schematic of generalized “molecular buoy” concept for pathogen isolation thorough buoyancy-assisted cell separation. Photograph of floated microspheres in buffer visually demonstrates separation of the hollow silica microspheres from the bulk solution.

Fig 2. Histogram plots depicting the size distribution of (A) H20 and (B) H50 microsphere diameters measured from phase-contrast microscopy images (inset).

Fig 3. (A) Photographs of H20 hollow silica microsphere floatation and separation at 30 sec intervals following mixing. (B) Kinetic traces from optical density measurements over 10 min for H20 microspheres in buffer solutions of increasing glycerol content (0-50%) were used to determine separation times in aqueous solutions with controlled viscosity. A reference line drawn at 0.9x max OD change in PBS buffer, 0% glycerol, illustrates the experimentally derived separation time in PBS. Separation times closely followed the exponential relationship between glycerol content and fluid viscosity (inset, red dashed curve); (C) Optical density curves of the H50 hollow-glass microspheres in buffer and glycerol. Baseline was buffer only, without microspheres.

Fig 4. Force diagram and equations describing the three forces acting upon the hollow microspheres in a suspended solution.

Fig 5. (A) Using wire mesh test sieves, four size-fractionated microsphere populations were obtained with mean diameters of (i) $21 \pm 5 \mu\text{m}$, (ii) $38 \pm 7 \mu\text{m}$, (iii) $62 \pm 10 \mu\text{m}$, and (iv) $81 \pm 12 \mu\text{m}$; (B) OD traces of each size-fractionated population in PBS and (C) 30% glycerol demonstrate rapid separation in roughly 2 min or less for all populations, except the $<20 \mu\text{m}$ dia. set, in aqueous solutions with viscosities comparable to biological fluids.

Fig 6. (A) Schematic of fully functionalized microspheres with protein G followed by a target-specific IgG antibody; (B) SEM micrographs of microspheres before functionalization (panel i) and after functionalization with protein G (panel ii); (C) Epi-fluorescent and bright-field images of fully functionalized microspheres at increasing concentrations of protein G (panels i-vi, 0 mg/ml control, 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml, respectively). Scale bar is 100 μm . Fluorescent images were brightness/contrast enhance for printing purposes only; (D) Quantitative mean fluorescence intensity (MFI) data fit to an exponential curve ($R^2 = 0.94$).

Fig 7. (A) Capture efficiency of the molecular buoys in buffer and stool samples spiked with *Cryptosporidium* oocysts. Significant differences using a one-tailed t-test are shown in brackets ($p < 0.05$); (b) Confocal z-slice image of two *Cryptosporidium* oocysts (white arrow) sandwiched between two hollow silica microspheres in a transmitted light overlay (top) and green fluorescence channel only (bottom).

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$$F_{buoyancy} = \rho_f V_f g \qquad \text{[Equation 1]}$$

$$F_{grav} = \rho_o V_o g = mg \qquad \text{[Equation 2]}$$

$$F_{drag} = 6\pi\eta r v \qquad \text{[Equation 3]}$$

Figure

(A)

(B)

(C)

Figure

Figure