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Author for correspondence:
Amy Q. Shen
e-mail: amyshen@uw.edu

†These authors are co-first author.

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Microfluidic one-step synthesis of alginate microspheres immobilized with antibodies

Wanyu Chen[1,4,†], Jong-Hoon Kim[1,†], Di Zhang[1], Kyong-Hoon Lee[1], G. A. Cangelosi[2], S. D. Soelberg[3], C. E. Furlong[3], Jae-Hyun Chung[1] and Amy Q. Shen[1]

[1]Department of Mechanical Engineering, [2]Department of Environmental and Occupational Health Sciences, and [3]Department of Medicine (Division of medical genetics) and Department Genome Sciences, University of Washington, Seattle, WA 98195, USA
[4]Wuhan University of Technology, Wuhan, Hubei 430070, People's Republic of China

Micrometre- and submicrometre-size functionalized beads are frequently used to capture targets of interest from a biological sample for biological characterizations and disease diagnosis. The main challenge of the microbead-based assay is in the immobilization of probe molecules onto the microbead surfaces. In this paper, we report a versatile droplet microfluidics method to fabricate alginate microspheres while simultaneously immobilizing anti-*Mycobacterium tuberculosis* complex IgY and anti-*Escherichia coli* IgG antibodies primarily on the porous alginate carriers for specific binding and binding affinity tests. The binding affinity of antibodies is directly measured by fluorescence intensity of stained target bacteria on the microspheres. We demonstrate that the functionalized alginate microspheres yield specificity comparable with an enzyme-linked immunosorbent assay. The high surface area-to-volume ratio of the functionalized porous alginate microspheres improves the detection limit. By using the droplet microfluidics, we can easily modify the size and shape of alginate microspheres, and increase the concentration of functionalized alginate microspheres to further enhance binding kinetics and enable multiplexing.

1. Introduction

Antibodies are produced by the immune system and are crucial for prevention and resolution of infection by foreign objects through highly specific interactions [1,2]. As a result, therapeutic antibodies have become very important treatment options [3]. Antibodies are also frequently identified or used for disease diagnosis due to their binding affinity and specificity [4]. Therefore, a rapid analysis of activity and binding properties of antibodies is essential to evaluate their performances. To characterize the binding affinity of antibodies, enzyme-linked immunosorbent assay (ELISA) has been routinely performed. As antibodies are sensitive to environmental factors such as pH and temperature, limited choices are available for immobilization of antibodies onto ELISA-based plate surfaces. Moreover, the ELISA format generally permits only a single analyte to be assessed in each well of a microwell plate, which limits multiplexing, assay flexibility, reliability and assay throughput.

As an alternative to ELISAs, microbead-based immunoassays are rapidly gaining popularity for several reasons [5–12]. First, multiplexed analysis is possible through immobilization of a multitude of antibodies onto a single bead or with combination of a number of beads carrying different antibodies. Second, bead-based immunoassays are more flexible, because the properties of beads can be modified with respect to their size and surface characteristics. In addition, the three-dimensional configuration of the microbeads can improve the access of target analytes to the bead surface [13,14]. Finally, microbeads can be manoeuvred with high levels of spatial- and temporal resolution under electrical [5,8], magnetic [7,11] and/or fluidic control [5,12]. Such flexibility facilitates highly sensitive detection with optimized parameters [15] and multiplexing [16].

The main challenge of the bead-based immunoassay is in the immobilization of antibodies onto the microbead surfaces. An immobilization procedure usually

affects the antibody activities [6]. Complex immobilization steps also require rigorous control of reagents and manufacturing processes. Immobilization of antibodies on solid microbeads can reduce the binding affinity of antibodies by damaging an antibody's three-dimensional molecular structure [17]. To address these challenges, hydrogels have become a desirable immobilization carrier for antibodies. Hydrogels are networks of hydrophilic cross-linked polymers that can undergo reversible deformation in response to external stimuli such as pH, salt concentration, temperature and electric fields, in the presence of aqueous or physiological fluids. Commercially available streptavidin-immobilized agarose beads can be coated with antibodies (Thermo Scientific Pierce). However, the antibody of interest is biotinylated (biotin attached to the antibody), involving very time-consuming procedures such as multi-step reactions, washings and centrifugations. Alginate, a natural, biocompatible and highly stable carbohydrate-based hydrogel, has been widely used as a drug carrier and encapsulation material [15] because it can be polymerized by simple gelation with divalent cations such as calcium ion [16]. Alginate-based immobilization methods in both film and sphere configurations have been reported with encouraging results [14,18,19], but the immobilization procedure is still rather complicated, involving multiple polymerization or conjugation steps for both hydrogels and antibodies, and multiple rounds of incubation and separation steps.

Microfluidics has emerged in recent years as a versatile method for manipulating fluids at small length scales, and, in particular, for generating micrometre-sized droplets with controllable size and functionality. Bacille Calmette–Guerin (BCG) contains a live attenuated (weakened) strain of *Mycobacterium bovis*. In this paper, we report a new strategy to immobilize anti-BCG IgY and anti-*Escherichia coli* (*E. coli*) IgG antibodies on alginate microspheres with a one-step droplet microfluidic method. More specifically, micrometre-sized sodium alginate droplets were first produced via a droplet microfluidics device, followed by external ion cross-linking set-up in a collection bath containing both calcium ions and antibodies in a buffer solution. Antibodies were immobilized primarily on the alginate microspheres during the gelation process. The binding affinity of antibodies can be directly analysed by fluorescence imaging of stained bacteria bound on the alginate microspheres. We also demonstrate that the functionalized alginate microspheres yield specificity comparable with an ELISA.

2. Material and methods

2.1. Materials

Calcium chloride and surfactant Span-80 were purchased from Sigma-Aldrich (St Louis, MO). Soya bean oil (viscosity = 50 mPa s^{-1}) was produced by Cibaria, Riverside, CA. Sterile sodium alginate (endotoxins < 100 EU g^{-1}) was purchased from Novamatrix, Oslo, Norway. Sylgard 184 silicone elastomer base and curing agent were purchased from Dow Corning Corporation. Tris-buffered saline (TBS, pH = 7.4) was purchased from Fisher Scientific. The anti-BCG polyclonal IgY antibodies were raised against *Mycobacterium tuberculosis* complex (*M. bovis* BCG) cells, and the anti-*E. coli* polyclonal IgG antibodies were purchased from ProSci Inc. (Poway, CA). To test the specific binding of bacterial cells on the alginate microspheres functionalized with antibodies, both BCG and *E. coli* cells at 10^7 CFU ml^{-1} in 1× TBS were stained with an intercalating dye (SYTO v. 9) green fluorescent nucleic acid stain (Molecular Probes L7007, Invitrogen, Carlsbad, CA). To eliminate unbound staining dyes, the solution was centrifuged to collect the pellet in a tube. The collected pellets were resuspended in TBS. The final concentration of the cells is approximately 10^6 CFU ml^{-1}. The SYTO 9 are generally used to label most bacterial cells with intact and damaged membranes. Both BCG and *E. coli* cells are typically rod-shaped, and are about 2 μm long and 0.5 μm diameter.

2.2. Fabrication of microfluidic devices

All the microfluidic devices were fabricated using standard soft lithography techniques by pouring poly(dimethylsiloxane, PDMS) pre-polymer along with cross-linker (pre-polymer: cross-linker = 10 : 1 by weight) onto a silicon wafer patterned with SU-8 photoresist. After degassing under vacuum in a desiccator for an hour, the PDMS material was baked for 2 h at 65°C in an oven. The PDMS replicas and glass slide were then bonded after oxygen plasma treatment and placed in an oven (65°C) for 2 days before experiments.

2.3. Structural analysis

After the alginate microspheres were collected on a glass coverslip, the sample was first frozen in liquid nitrogen and dried under vacuum. The dried sample was then coated with gold and characterized by scanning electron microscopy (SEM Sirion, FEI, 5 kV).

2.4. Analysis of binding affinity

The antibody-coated alginate microspheres were prepared in 1× TBS with anti-BCG IgY (1.8 mg ml^{-1}) and anti-*E. coli* IgG (0.5 mg ml^{-1}). We consider these concentration values as the upper limit of antibody concentrations in our studies. If the antibody concentration is very high, antibodies can aggregate and overlap with each other and lower their functionality. If the antibody concentration is very low, the binding affinity can be similar to that of bare alginate microgels, hence the probability of binding events is reduced. To visualize individual cells, BCG cells (or *E. coli* cells) were stained with the intercalating dye (SYTO v. 9 green fluorescent nucleic acid stain; Molecular Probes L7007) in 1× TBS. The stained BCG cells (or *E. coli* cells) were mixed with the alginate microspheres and incubated for 15 min. Subsequently, a 2 μl droplet of the mixture was placed on the glass slide for imaging under an epifluorescence microscope (Olympus BX-41, Olympus America Inc., Melville, NY). To quantify the results, we randomly picked 18 fluorescence images from the mixture and divided them into six groups. Each group consisted of three images. From each group, the total number of the microspheres and the number of microspheres bound to cells were counted. The latter was then divided by the former to calculate the binding probability.

2.5. ELISA test for anti-BCG IgY and anti-*Escherichia coli* IgG

Equal concentrations (OD_{600} matched) of two bacterial strains (BCG and *E. coli* of 10^6 CFU ml^{-1} in 100 μl of phosphate-buffered saline (PBS) each) were assayed for binding to anti-BCG IgY antibodies and anti-*E. coli* IgG antibodies using a 0.45 μm filter plate (Millipore Billerica, MA, no. MAHVN4510). Aliquots of the bacterial suspensions were added to the 96-well filter bottom plate and washed with PBS. Subsequently, a 100 μl aliquot of 10 μg ml^{-1} IgY anti-BCG or IgG-anti-*E. coli* antibodies in PBS were added to the washed cells and incubated for 1 h at 37°C. After another PBS wash, a secondary antibody was added (rabbit anti-IgY-HRP conjugate, Thermo Scientific, no. 31401, or goat anti-Rabbit IgG, Thermo Scientific, no. 31460) and

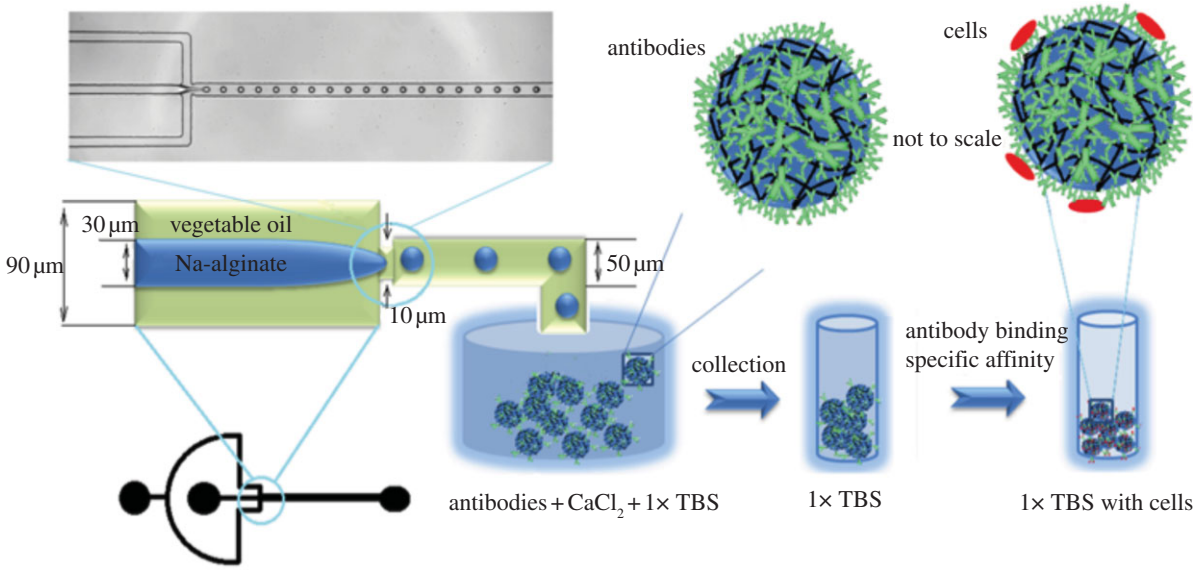

Figure 1. The droplet microfluidics and external gelation set-up for fabricating alginate microspheres with immobilized antibodies. Target bacteria are bound with the functionalized microspheres for specificity tests. The top left image is a snapshot of the sodium alginate droplet of 30 µm diameter generated from the flow focusing microchannel, moving downstream. (Online version in colour.)

incubated for 1 h at 37°C. The sample was then washed again with PBS, followed by addition of 100 µl of ABTS (2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate (Thermo Scientific, no. 37615), incubated for 5 min, and filtered into a clear 96-well receiver plate. Absorbance was then recorded at 405 nm.

3. Results and discussions

3.1. Immobilizing antibodies on alginate microgels

The one-step droplet microfluidics devices were used to immobilize antibodies on alginate microspheres as shown in figure 1. A flow focusing design (the channel depth is approx. 75 µm) involving two immiscible fluids was used to produce sodium alginate (Na-alginate) droplets. The aqueous Na-alginate solution (1 wt%) was used as the dispersed phase (in blue) and soya bean oil containing surfactant Span 80 (5 wt%) was used as the continuous phase (in green). Both solutions were pumped into the microchannel by digital syringe pumps (Harvard Apparatus). Owing to the competition between viscous and capillary forces, a thread of Na-alginate solution periodically broke up to discrete droplets under certain flow rates of the two phases [20]. The droplet size can be controlled by the flow rates of the two phases, the viscosity ratio and the interfacial tension of the two phases. The droplet size decreases with increasing flow rate of the oil phase or with decreasing flow rate of the aqueous alginate phase. Droplet size ranging from a diameter of 25 to 50 µm is the target size of this work.

Resultant Na-alginate droplets (the diameter approx. 50 µm with 250 µl h^{-1} for the oil phase and 50 µl h^{-1} for the dispersed aqueous phase) were collected from the outflow into a vial of 1× TBS consisting of 0.1 g ml^{-1} $CaCl_2$ and antibody (1.8 anti-BCG IgY or 0.5 mg ml^{-1} anti-*E. coli* IgG). The outflow tubing touched lightly on the surface of the collection bath. The droplets were subsequently cross-linked by Ca^{2+} in the buffer solution to form alginate microgels (figure 2*a*,*b*). In our system, Na-alginate is a sodium salt of alginic acid, a natural polysaccharide and a linear polymer composed of 1,4-linked β-D-mannuronic acid (M) and α-D-gluronic acid (G) residues in varying proportions and arrangements. Sodium alginate is water-soluble and forms a reticulated structure that can be cross-linked with divalent or polyvalent cations to form an insoluble meshwork. In our work, calcium cations are used to cross-link with the acid groups of alginate to form ionotropic gels [21].

Simultaneously, the antibodies were immobilized on the porous network of the alginate microsphere during the external cross-linking process (figure 1). To prevent potential aggregation from the functionalized alginate microgels, the vial was placed on a reciprocating shaker (Thermo Scientific) during the synthesis process, with gentle motion at 10 rpm with 1 inch stroke length. We did not observe any microgel aggregation at the particle concentration of approximately 160 particles mm^{-2} based on fluorescence microscope images, and most microgels maintained spherical shapes, with some small percentage being slightly deformed (figure 2*b*). The alginate gel microspheres (final diameter ranging 50–60 µm) were then centrifuged at 800*g* for 5 min to separate the oil from the alginate microspheres with the immobilized antibodies, prior to the binding affinity measurements. Two buffer solutions are routinely used for antibodies: TBS and PBS. Because $CaCl_2$ can react with HPO_4^- contained in the PBS to induce precipitation and aggregation of $CaHPO_4$, we used TBS in all of the experiments.

Figure 2*a* shows the optical microscopy image of the alginate microgels. Figure 2*b* shows the fluorescence image of alginate microspheres (diameter: 60 µm) with fluorescein-labelled anti-BCG antibodies, captured at mid-plane. The brightest colour at the outer rim of the microsphere corresponds to the fluorescein-labelled antibodies. While the darker and non-uniform colours inside the sphere correspond to the interior region and the shadows projected from the bottom of the three-dimensional sphere.

Figure 2*c*,*d* shows the SEM images of the alginate microgels. The cracks were caused by sample preparation when the alginate microspheres were frozen in liquid nitrogen and then dried under vacuum before imaging. Based on a series of SEM images, the alginate microspheres contain a porous

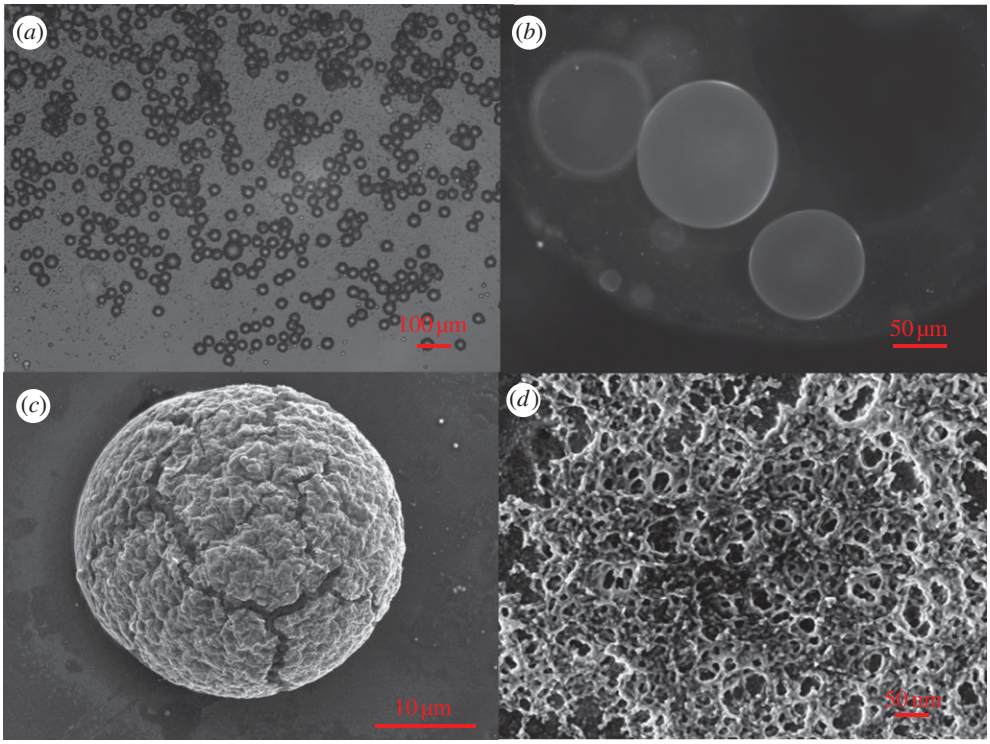

Figure 2. (*a*) Alginate gel microspheres (diameter: 30 µm) with immobilized antibodies; (*b*) fluorescence image of alginate microspheres (diameter: 60 µm) with fluorescein-labelled anti-BCG antibodies, captured at mid-plane, stored in 1× TBS solution. The outer rim of the sphere (in the bright colour) represents the surface coating of the antibodies, whereas the region inside the sphere represents the interior of the microbead; (*c*,*d*) scanning electron microscopy (SEM, Sirion, FEI, 5 kV) images of an alginate microsphere. Note that smaller-sized microbead is selected for SEM imaging. (Online version in colour.)

network with pore size ranging between 5 and 100 nm, with the dominant pore size ranging from 30 to 50 nm (see the electronic supplementary material, figure S4). Because polyclonal antibody size ranges between 10 and 20 nm, the binding between the antibody and the alginate will be dominated by capillary and electrostatic interaction. Hence, as long as the pore size of the alginate is larger than 10–20 nm, there is a good chance that antibody can bind with the alginate, so that neither conjugation nor covalent linkage between the carrier beads and the antibodies is required. Therefore, the alginate beads fully bound with antibodies can offer higher chances for binding with target bacteria.

In addition, because the calcium ions were abundant in the collection bath, the alginate microgels carried positive calcium ions even after we washed the solidified alginate microgels with TBS. To verify the charge of alginate microspheres in the TBS, we used planar electrodes by applying a DC potential. When a DC potential was applied, alginate microgels were aggregated on the negative electrode, indicating that alginate beads are positively charged in the TBS (see the electronic supplementary material, figure S1). As a result, the slightly negatively charged antibodies (isoelectric point less than 6.5) can bind with the positively charged alginate microspheres by electrostatic attraction (i.e. Coulomb force) during the cross-linking process. In short, both the nanoporous structure and the surface charge of the alginate microgels provide a simple platform to immobilize the antibodies, with high throughput and yield.

Note that the antibodies can be immobilized with the alginate microgels when they are mixed either with the Na-alginate solution in the microfluidic device or in the collection bath with $CaCl_2$ in the buffer solution. The former approach showed less optimal results owing to (i) flow conditions in the microfluidic device can exert undesirable stress on the antibodies, and (ii) immobilized antibodies mainly resided inside the alginate microgels instead of the surface, because the cross-linking process is initiated at the interface of the Na-alginate and the buffer solution containing $CaCl_2$. To ensure maximum and more uniform surface coverage of the antibody on the alginate microgels for future detection purposes, we adopted the latter procedure as shown in figure 1.

To identify the extent of the surface coverage of the immobilized antibodies on the alginate microgels, alginate microbeads immobilized with fluorescein-conjugated antibodies were prepared and observed under the fluorescence microscope (figures 2*b* and 3). We further examined the fluorescence intensity profile along three focal planes (top, middle and bottom) of the alginate microbead (see the electronic supplementary material, figure S5). Figure 3 shows the fluorescence intensity profile in the middle focal plane of an alginate microbead immobilized with fluorescein-conjugated antibodies. The area showing the higher fluorescence intensity indicates more densely immobilized fluorescein-conjugated antibodies. Based on five randomly selected functionalized alginate microspheres and characterizing their fluorescence intensity along all three focal planes, we conclude that antibodies are mainly immobilized on the surface of the alginate microbead with some small percentage entrapped inside the alginate microsphere. Note that only the outer rim of the sphere (brightest colour) represents the surface coating of the antibodies, whereas the region inside the spherical image represents the interior of the microbead.

3.2. The stability of functionalized alginate microspheres

We also investigated the stability of functionalized alginate microspheres. We used fluorescein-labelled anti-BCG antibodies to study the stability of the antibodies immobilized on

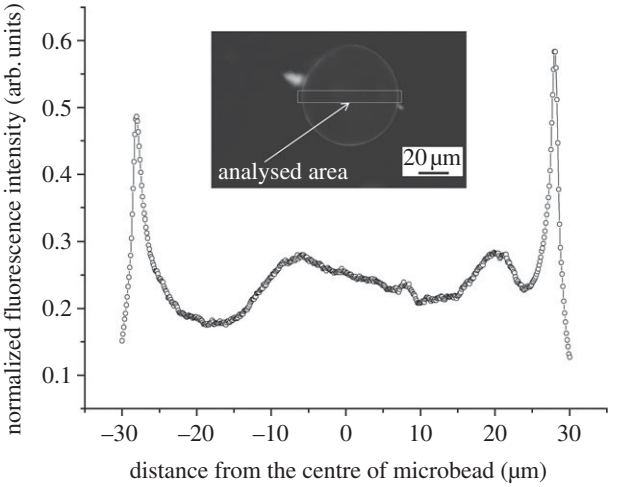

Figure 3. Fluorescence intensity profile in the middle focal plane of an alginate microbead immobilized with fluorescein-conjugated antibodies. White dots exhibit the highest fluorescence signal. Inset: a representative fluorescence image for alginate microbead coated with fluorescein-conjugated antibodies. More images captured at different focal planes can be found in the electronic supplementary material, figure S5.

the alginate microgels. We used a vial of 0.1 g ml^{-1} $CaCl_2$ with 0.2 mg ml^{-1} fluorescein-labelled anti-BCG antibodies in 1× TBS to collect the sodium alginate microdroplets exiting the microfluidic device. After these antibody-immobilized microspheres were separated from the oil and suspended in 1× TBS solution, we placed a 2 µl suspension on the glass slide and observed the sample under a fluorescent microscope (Nikon) on days 0, 1, 2, 5, 10, 20 and 30. The antibodies were stored at 4°C during the 30 day period. The average intensity of individual beads was evaluated by using ImageJ (NIH) and normalized with the background. The intensity is correlated linearly with the antibody stability.

Figure 4 shows the fluorescence intensities and densities of the alginate microspheres after the microspheres immobilized with antibodies maintained in 1× TBS solution for 0, 10 and 30 days, respectively. To normalize the fluorescence signal, the intensity profile of the microbead was measured and divided by the maximum value identified from all experiments (total of 42 microbeads). Initially, the normalized fluorescence intensity of alginate microspheres was 2.02 (figure 4*a*). At day 1 and day 2, the normalized fluorescence intensity became 1.99 and 1.96, respectively. The intensity did decrease slightly over time. In particular, the fluorescence intensity maintained its original level for the first 5 days. Additional fluorescence images of the alginate microspheres immobilized with fluorescein-labelled antibodies at days 0, 1, 2, 3, 4, 5 are shown in the electronic supplementary material, figure S3. At day 30, the normalized fluorescence intensity dropped to 1.73 (a 14% decrease from the initial value). Nevertheless, all error bars overlapped between the intensities for the 30 day period. Considering the high concentration (1.8 mg ml^{-1}) of anti-BCG IgY antibodies immobilized on and in the alginate microspheres, 14% reduction of the fluorescence intensity is acceptable. Overall, our results show that immobilized antibodies are quite stable for 30 days at 4°C.

We also observed that the number of the functionalized alginate microbeads decreased over a period of 30 days, even though the fluorescence intensity of each individual alginate microsphere did not vary much over time. As shown in

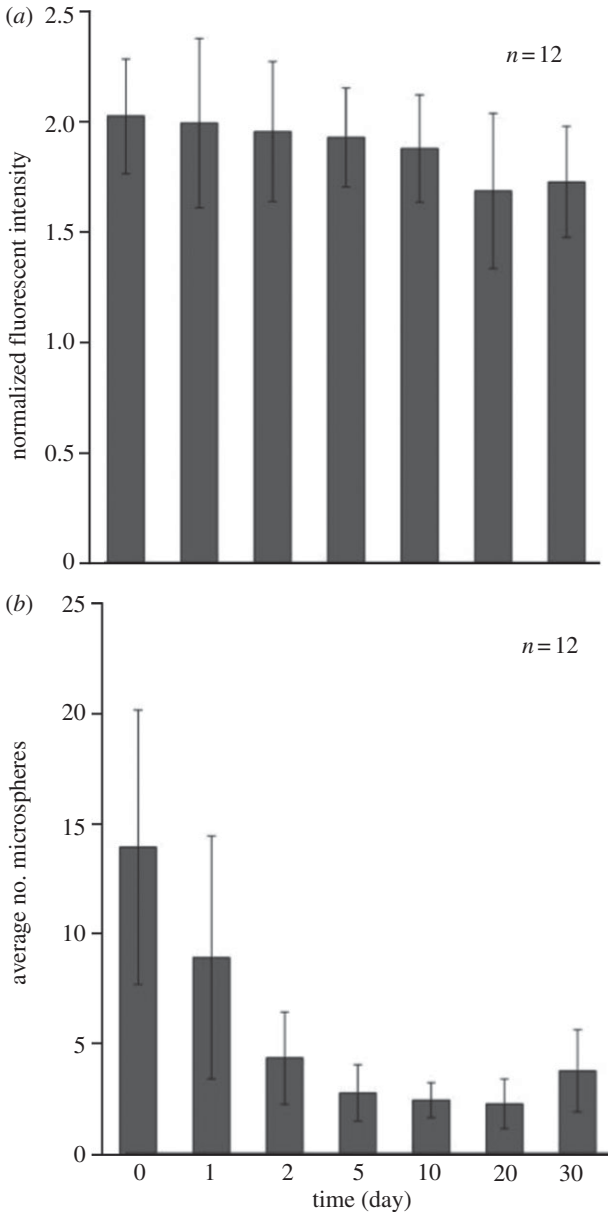

Figure 4. (*a*) The normalized fluorescent intensities of functionalized alginate microspheres over 30 days; (*b*) the average number of alginate microspheres over 30 days.

figure 4*b*, at days 0, 1 and 2, the number of functionalized alginate microgels in a 2 µl TBS suspension were 14, 9 and 4, respectively. The number of alginate microspheres reached a plateau after day 2. The number of functionalized alginate microspheres decreased due to the swelling and merging of some individual alginate microspheres, which could have caused the reduction of the fluorescence intensities of the functionalized alginate microbeads over time. In the electronic supplementary material we show the swelling rate of the alginate microspheres with fluorescein-labelled anti-BCG antibodies when they were suspended in 100 ml 1× TBS at 4°C. To prevent the swelling and enhance the long-term stability of functionalized alginate microbeads, a number of additives can be considered to modify our existing alginate recipe. Carboxymethyl chitosan is a natural polymer that has a number of commercial and possible biomedical uses [22]. Poly-L-lysine is non-toxic and can be used as a drug carrier [23]. PEG200 is non-toxic and it has been used in toothpastes [24]. All these additives should improve the stiffness and

reduce the swelling of our current alginate microbeads. The results of the modified alginate recipe will be reported in the near future.

3.3. Specific and non-specific binding to alginate microspheres

The binding affinity of the functionalized microspheres was analysed by binding target cells to the alginate microspheres functionalized with their corresponding antibodies. The binding events can include both non-specific and specific bindings. Non-specific binding can be generated by electrostatic, hydrophobic or hydrophilic, or van der Waals interactions, which are usually present in the immobilization process. Specific binding of antibodies can be explained by association and dissociation constants. For immunocomplex formation, the association constant k_a is 10^7 s M^{-1}, whereas the dissociation constant k_d ranges from 10^{-5} to 10^{-2} s^{-1} [25]. Because the association constant is similar for immunocomplex formation, the binding events between the antibodies and antigens are differentiated by the dissociation constants. Thus, the specific binding reaction is limited by diffusion and reaction constants. The probability of binding increases for the target cells, whereas the probability decreases for the binding of non-target cells.

In our experiments, both non-specific and specific binding of microspheres were examined by incubating functionalized alginate microspheres and bacterial cells for 15 min at room temperature. The two bacteria cells, *Mycobacterium bovis* BCG (BCG; 10^6 CFU ml^{-1}) and *E. coli* (10^6 CFU ml^{-1}) were prepared. The corresponding polyclonal antibodies, anti-BCG IgY and anti-*E. coli* IgG, were also prepared. The experimental studies are summarized in table 1. To observe the binding events, cells were stained with an intercalating dye (SYTO v. 9 green fluorescent nucleic acid stain, Molecular Probes L7007). The stained cells were visualized by an epi-fluorescence microscope (Olympus BX-41). After we mixed stained BCG cells and *E. coli* cells with the alginate microspheres, we counted the cells bound with the antibodies in the functionalized alginate microsphere carrier for each case.

Specific binding of BCG and *E. coli* cells with alginate gel microspheres immobilized with anti-BCG IgY was examined and imaged. When the alginate microspheres were functionalized with specific antibodies, the target bacteria stained with green fluorescence dyes were bound to the functionalized alginate microspheres as shown in figure 5. For alginate microspheres functionalized with anti-BCG IgY antibodies, 61% of alginate microspheres bound BCG cells (figure 5*a*), but only 13% bound *E. coli* (figure 5*b*). When the antibodies were switched to anti-*E. coli* IgG, 78% of the microspheres bound *E. coli* (figure 5*c*) and 41% showed cross reactivity (non-specific binding) with BCG (figure 5*d*). By immobilizing anti-*E. coli* IgG on the alginate microspheres, the binding affinity was more specific for *E. coli* cells. However, non-specific binding for BCG was still observed on the alginate microbeads immobilized with *E. coli* antibodies.

With alginate microspheres being slightly positively charged on the surface, and BCG and *E. coli* cells being negatively charged [26,27], non-specific bindings can occur through electrostatic interactions. By measuring the contact angle of the bacteria cell suspension in TBS solution on the functionalized alginate film, we observed that the contact angle of an aliquot of BCG in TBS was smaller than that of *E. coli*, which indicates that the surface interaction between the BCG suspension and the alginate is stronger, meaning BCG cells are more 'sticky' towards alginate material in comparison with that of the *E. coli* cells. Future work will focus on decreasing the non-specific binding between BCG cells and the functionalized alginate microspheres. We can adopt similar procedures for non-specific binding reduction routinely performed in ELISAs. For example, we can add 1% bovine serum albumin or casein solutions, along with adding 0.1% Tween 20 in the binding buffer solutions.

Table 1. Alginate microspheres bound with different cells.

alginate microspheres	cells	bindings
immobilized with anti-BCG IgY	BCG	specific
	E. coli	non-specific
immobilized with anti-*E. coli* IgG	BCG	non-specific
	E. coli	specific

To compare the binding affinity of the antibodies with ELISA, the specificity of anti-BCG and anti-*E. coli* antibodies was also evaluated using an ELISA kit. Overall, the specificities by ELISA qualitatively agreed with those by the functionalized alginate microbeads approach (figure 6). The alginate microsphere approach measures the binding affinity by counting individual binding events, whereas an ELISA method measures the average intensity of signals from the binding events. In the experiments shown here, we used similar concentrations of bacteria of 10^6 CFU ml^{-1}. However, the ELISA format exhibited somewhat less variability and greater specificity than the microsphere format when the BCG cells are involved (table 2 and figure 6). In comparison with ELISA method with anti-BCG antibody, the alginate microbead approach performs better for the specific binding case, with similar non-specific binding performance.

The higher affinity of BCG cells for the alginate microspheres is partially attributed to the non-specific binding between BCG cells and alginate microspheres. Because inherent non-specific binding is highly dependent on the cell properties, and inherent association and dissociation events between the antibodies and the cells, one strategy is to modify the existing alginate recipe (see the stability discussion above) to enhance the stiffness of alginate microspheres while reducing the non-specific binding activities. Considering that both ELISA and microsphere methods are based on diffusion-limited reactions, the specific binding of the microbead assay may also be enhanced by stirring the sample solution. Nevertheless, our studies show that the binding affinity of antibodies in bacteria suspensions by the functionalized alginate microspheres is comparable with the ELISA results. Moreover, our alginate microbead approach offers potentials to add additional biological, magnetic and electrical properties in the functionalized microbeads with our droplet microfluidic set-up, which can significantly reduce the barrier of conducting rapid and simple diagnosis of diseases.

4. Conclusions

In summary, we developed a simple droplet microfluidic method to immobilize the antibodies in porous alginate

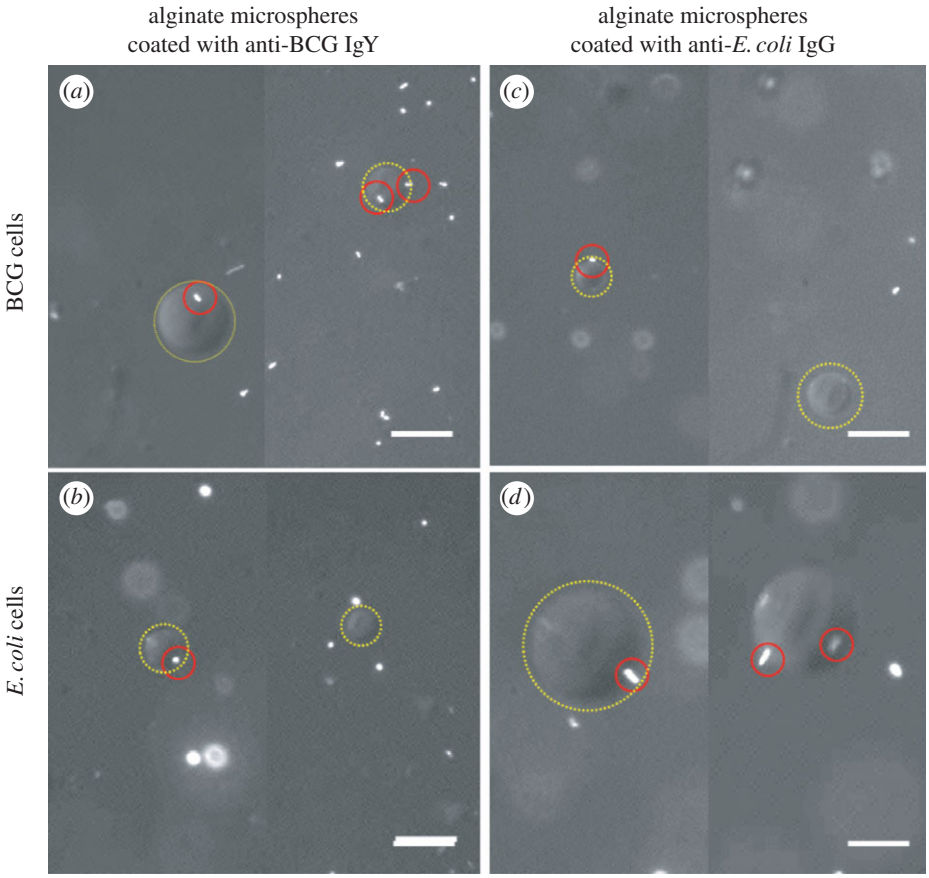

Figure 5. (*a–d*) The fluorescent images of functionalized alginate microspheres bound with BCG and *E. coli* cells. BCG and *E. coli* cells are shown by white dots. We show two representative images in each case. The concentration of BCG and *E. coli* is 10^6 CFU ml^{-1}. The scale bar is 20 μm. (Online version in colour.)

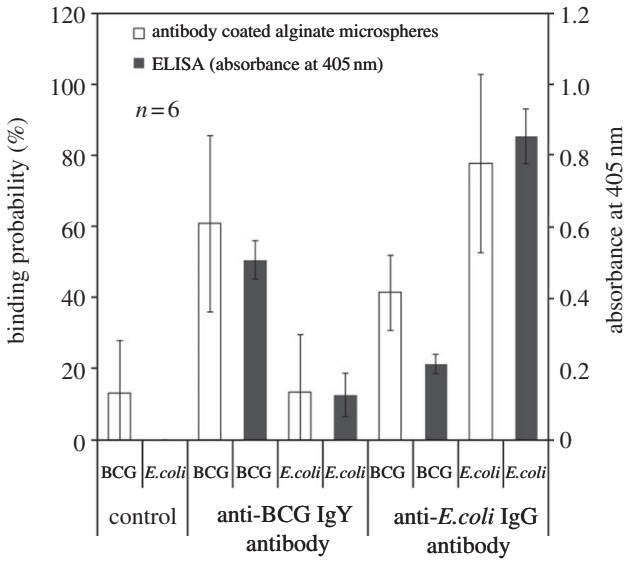

Figure 6. Binding probability of BCG and *E. coli* cells on the alginate microspheres, immobilized with anti-BCG and anti-*E. coli* antibodies, in comparison with ELISA measurements.

microspheres (diameter ranging from 40 to 60 μm). Our results demonstrated that immobilized antibodies maintained the binding affinity in the hydrated state. Owing to the large surface area of the nanoporous alginate microgels, the binding probability between the target bacteria and the immobilized antibodies was enhanced. Furthermore, the binding affinity of antibodies could be directly analysed by fluorescence imaging of stained bacteria on the microspheres. The binding of functionalized alginate spheres showed the specificity comparable with that of an ELISA. In comparison with ELISA, the alginate microbead approach involves simpler operating procedures with lower costs. However, future work is required to reduce non-specific interactions between the target bacteria and alginate microspheres to further improve the specificity. With the optimized sizes and shapes of alginate microbeads, the binding probability with target cells can also be enhanced. By immobilizing different antibodies on the alginate microgels and mixing them in the specific affinity test, we can potentially achieve multiplexing. With these advantages, the alginate microsphere approach illustrated in this work, can be implemented for affinity binding tests, disease diagnosis, targeted drug delivery and drug discovery.

Table 2. Binding affinity tests with microbead and ELISA methods.

	anti-BCG IgY		anti-*E. coli* IgG	
methods	BCG	*E. coli*	BCG	*E. coli*
alginate microbeads (binding probability, %)	61	13	41	78
ELISA (absorbance 405 nm)	0.51	0.13	0.21	0.85

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