QUANTITATIVE ESTIMATION OF THE LIPID PRODUCTIVITY OF SINGLE ALGAE CELLS IN ALGINATE HYDROGEL MICROBEADS

Do-Hyun Lee[1]*, Chae Yun Bae[1], Jong-In Han[2], and Je-Kyun Park[1]
[1]Department of Bio and Brain Engineering, KAIST, REPUBLIC OF KOREA
[2]Department of Civil and Environmental Engineering, KAIST, REPUBLIC OF KOREA

ABSTRACT

This paper presents the first demonstration of the droplet-based microfluidics to fabricate an alginate hydrogel microbead with a diameter of ~30 μm that encapsulates single microalga from three species of green microalgae (*Chlorella vulgaris*, *Chlamydomonas sp.*, and *Botryococcus braunii*) for application to the real-time screening of single cells. We performed the quantitative estimation of the lipid content of individual microalgal cells by non-destructive fluorescence staining using BODIPY. This feature leads to easy combination of a high-throughput screening system which could be applicable to the economically feasible production of microalgae for biofuels.

KEYWORDS

Microalgae, Droplet-based microfluidics, Alginate hydrogel, Microbead, Lipid measurement

INTRODUCTION

Biofuels, which includes biodiesel from microalgae, have attracted particular interest for a potential alternative of fossil energy due to the considerable variations in lipid composition [1]. To achieve the commercial benefit of microalgae-based biofuel production, isolation of algae species containing abundant lipid species with high-resolution analysis is required [2,3]. On the basis of literature data, the fatty acid profiles have identified consistently with the recent experimental reports, but there was an individual difference among the algae cells from the same species under equal environmental conditions. On the other hand, there is some heterogeneity with regard to the lipid accumulation among the individual cells in each species. For this to be effective, it is essential that individual cells of each species should be encapsulated within a microchamber in a single cell level [4].

A microfluidic method can achieve rapid production of highly monodispersed droplets in micro-sized diameter, and thus we can construct a nanolab with cell-like environment which accommodates a single cell [5,6]. An advantage of droplet-based microfluidics is that the isolation, manipulation, detection, retrieval and collection of single cells can be easily achieved with an increased throughput and a decreased level of complexity [7]. Especially, hydrogel is a promising material for cell encapsulation which allows a high degree of permeability for continuous supplying of nutrients and maintenance of cell viability [8,9]. Here we first demonstrate the droplet-based microfluidics to fabricate an alginate hydrogel microbead that encapsulates single algae cells for application to the real-time screening of single cells. The additional calcified oil flow *via* the microbridge structures promoted droplet spacing increment and rapid gelation of microbeads with high stability. The combination of BODIPY, a non-destructive stain for lipid determination in live algal cells, was utilized for the quantitative comparison of their lipid content of individual cells within the microbead.
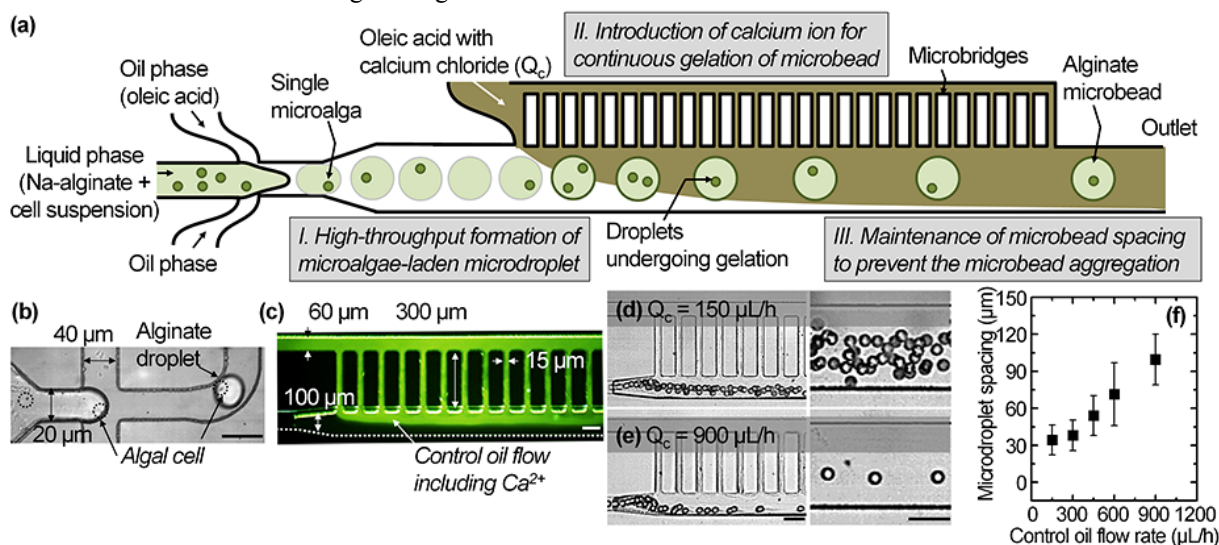

Figure 1: (a) A schematic of the microfluidic device for generation alginate microbeads containing algal cells with sufficient spacing. (b) A bright-field microscopy image showing cell encapsulation into an alginate droplet at the cross junction. (c) A fluorescence image illustrates channel design of a microfluidic device with microbridge structures. (d,e) Microscopic images of varying droplet interval within main channel and nearby the outlet when Q_c is (d) 150 μL/h and (e) 900 μL/h. (f) Plot of the microdroplet interval with respect to the Q_c. Scale bars: 50 μm.

EXPERIMENTAL

Working principle

Figure 1a shows the schematic of the microfluidic device and its adjusting interval and gelation of alginate droplets [10]. We designed the microbridge structures bridging the droplet-generating and control channels with additional inlet to make a pressure drop between two microchannels. The microbridge plays a role in microbead preparation such as the prevention of the aggregation of surrounding microbeads by suitable microfluidic spacing, and the maintenance of the droplet size. In this experiment, we encapsulated single cells from three species of green microalgae (*Chlorella vulgaris, Chlamydomonas sp.* and *Botryococcus braunii*). We dispersed microdroplets of Na-alginate in cell media containing an algae cell in a continuous phase of oleic acid with surfactant (4% w/w) by flow focusing method at a cross junction (Figure 1b). In order to form spherical hydrogel microbeads, the droplet shape could be recovered from plug to sphere due to the enlarged channel height from 35 to 100 μm. Further downstream, the additional control oil flow (Q_c) including calcified oleic acid (24 mg/mL) with surfactant changes the velocity of the main flow *via* the microbridge structures, similarly described in our previous work [2] (Figure 1c). Figure 1d and 1e shows the microscopic images varying droplet interval within microchannel and nearby the outlet with respect to the Q_c is 150 and 900 μL/h.

Device Design and Fabrication

A microfluidic chip integrated with microbridge structures was fabricated in poly(dimethylsiloxane) (PDMS) by soft lithography, as shown in Figure 1c. The widths of the Na-alginate and oil channels were 20 and 40 μm, respectively. To ensure complete interval control of droplets, we used the microdevice with 45 microbridges, whose width and length were 15 and 300 μm, respectively, and main channel and diverging microchannel nearby the outlet whose widths were 100 and 200 μm, respectively. We fabricated the mold structures with photolithography in SU-8 photoresist (SU8-2025; MicroChem Corp., MA, USA). All fluidic channel and microbridge structure were fabricated from PDMS silicon elastomer (Sylgard 184; Dow Corning, Midland, MI, USA) which was mixed with a curing agent in a ratio of 7:1 (w/w) using standard soft lithographic methods. The PDMS microchannel was then irreversibly bonded by plasma activation on a glass slide. Because the hydrophobic PDMS microchannel became hydrophilic during plasma treatment, the chip was stored in a convection oven at 65 °C for 2 days to recover hydrophobic nature of the microchannel. After the fabrication process, the microfluidic devices were sterilized with 1% (w/v) Pluronic F127 in distilled water prior to cell loading without algal cell attachment. Subsequently, the microchannels were flushed with an oil phase.

Material Preparation

Sodium alginate (A0682-100G; Sigma) was dissolved in JM media by 1% (w/w) and filtered with a 0.22 μm syringe filter (Millex-GV; Millipore) to remove any clumps of alginate. Oleic acid (Sigma) with Abil EM 90

surfactant (4% w/w) was introduced as a continuous phase and the Na-alginate was crosslinked by the calcified oleic acid (24 mg/mL). The calcified oleic acid was prepared as follows: 0.6 g of calcium chloride (C7902-500G; Sigma) was dissolved in 25 mL of oleic acid via ultrasonication. Calcium chloride was dissolved in 25 mL of 2-methyl-1-propanol (J.T. Baker, Deventer, The Netherlands) via ultrasonication. After mixing calcium chloride and oleic acid at a ratio of 50% (v/v), the 2-methyl-1-propanol was distilled in a convection oven at 65 °C for a day.

C. vulgaris was cultivated at 20 °C in non-salinity BG 11 medium. *Chlamydomonas sp.* (KMMCC-1681) and *B. braunii* (KMMCC-868) were obtained from the algal culture collection at the Korean Marine Microalgae Culture Center (Busan, Korea). *Chlamydomonas sp.* and *B. braunii* were grown in JM medium prepared in sterilized natural freshwater. All cultures were conducted under constant shaking with an agitation speed of 120 rpm and continuous illumination with a 3000 lux intensity lamp. BODIPY 505/515 (Invitrogen Molecular Probes, CA) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 100 mg/L. An incubation time of 30 min in darkness at room temperature was used for all staining process.

RESULTS AND DISCUSSION

As the Q_c increased, the droplet interval was significantly increased without any effect on the droplet generating pattern (Figure 1g). When Q_c was adjusted from 150 to 900 μL/h, the droplet interval was changed from 34.40 ± 12.14 to 99.52 ± 20.48 μm, at a fixed condition of droplet generation with a Na-alginate flow rate (Q_w) of 20 μL/h and oil flow rate (Q_o) of 50 μL/h. Under the relatively lower Q_c, the undesired coalescence hydrogel microbead was observed (data now shown). The stable formation of cell-laden alginate hydrogel microbeads was guaranteed by the suitable spacing between two approaching microdroplets. This unwanted aggregation of hydrogel microbeads due to insufficient spacing lowers monodispersity and single-cell encapsulation efficiency.
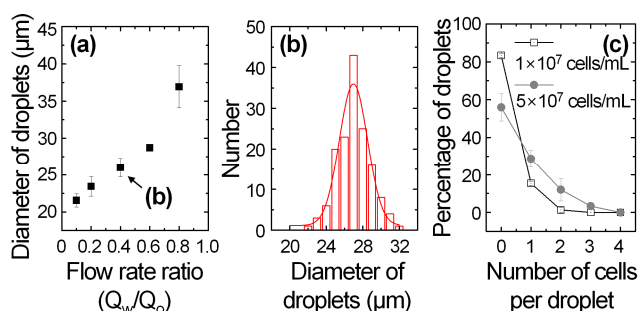
Figure 2: (a) Plot of the droplet diameter with respect to the flow rate ratio (Q_w/Q_o, Q_o=50 μL/h). (b) The diameter distribution of alginate droplets shown under the flow condition of Q_w = 20 μL/h and Q_o = 50 μL/h. (c) Percentage of single-cell containing droplets at different initial loading concentration of 1×10^7 and 5×10^7 cells/mL. In the proposed device, we were able to obtain up to 35% of droplets having single algae cells inside. Scale bars: 20 μm.

Figure 2a shows the variation of droplet diameter with the flow rate ratio (Q_w/Q_o; Q_w refers to the water flow rate) when the oil flow rate (Q_o) is 50 µL/h. Figure 2b shows the diameter distribution of alginate droplets with *C. vulgaris* shown under the flow condition of $Q_w = 20$ µL/h and $Q_o = 50$ µL/h. The microbead diameter could be adjusted from 22 to 37 µm. As shown in Figure 2b, we could accurately produce monodisperse algae cell-encapsulating alginate droplets with a mean diameter of 26 µm. We also confirmed the percentage of single-cell containing droplets according to different initial cell loading concentration (1×10^7 and 5×10^7 cells/mL). It was very similar to the theoretical value of Poisson distribution. In the proposed device, we were able to obtain up to 35% of droplets having single algae cells inside (Figure 2c). The efficiency of single-cell encapsulation would be enhanced by incorporating the interconnection technique or the secondary microfluidic networks [11].
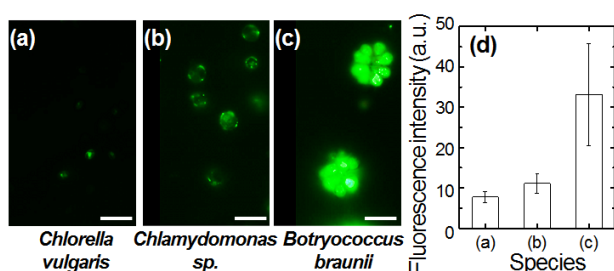

Figure 3: Fluorescence images of (a) C. vulgaris, (b) Chlamydomonas sp., and (c) B. braunii, respectively stained with BODIPY in bulk scale. Scale bars: 20 µm. (d) The average fluorescence intensity of each of the three microalga in the microbeads (n = 60).

Figure 3a–c shows the BODIPY-stained fluorescence images of three species of algae in bulk scale. After staining with BODIPY, the lipid bodies in all three algal cells indeed exhibited bright green fluorescence, although fluorescence intensity greatly varied. We also confirmed that the lipid accumulation of a single cell of among three species can be quantitatively estimated by the direct observation of the green fluorescence within the microbead by using a fluorescence microscope (data not shown). Although the cell was enclosed into the microbeads, the amount of intercellular lipids in an algal cell was also detectable. Also, there was a significant difference in fluorescence intensity among three species (Figure 3d) due to the difference of the lipid content within the cells. In particular, *B. braunii* has relatively high lipid content than other species of *C. vulgaris* and *Chlamydomonas sp.*

Compared to Nile Red, a well-established dye for detecting the lipid content of animal cells and microorganisms, is unable to guarantee the cell viability. Its emission spectrum that shares with that of chlorophyll also interferes with the accurate estimation of intercellular lipids. BODIPY is enough to remedy the disadvantages that previously mentioned [12], thus we could estimate the lipid content of individual microalga by combination of BODIPY staining in the microfluidic device. In all cases, we confirmed that the lipid content of microalgal species in

alginate hydrogel microbeads was comparable to that of freely living cells. In future, automated or interactive selection of the microbead containing a desired alga without complicated apparatus is strongly required in the fields of environmental technologies [13].
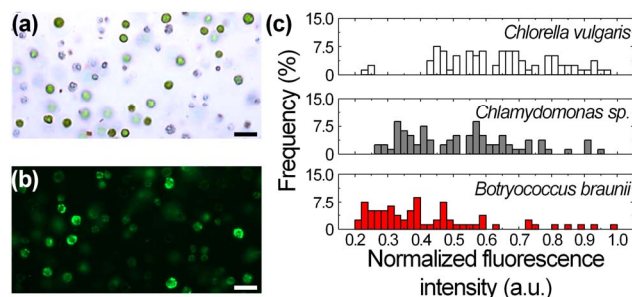

Figure 4: (a) Bright-field and (b) fluorescence images of Chlamydomonas sp. stained with BODIPY in bulk scale to show the heterogeneity in the lipid accumulation among the individual cells. Scale bars: 20 µm. (c) Histograms showing the distribution of green fluorescence among individual cells in three species of algae.

Due to the variability in cell status based on their growth rate, there is some heterogeneity with regard to the lipid accumulation among the individual cells in each species. We also confirmed the difference in lipid accumulation among individual cells of the same species (Figure 4a and 4b) based on the BODIPY fluorescence intensity. Figure 4c presents a histogram which shows the distribution of green fluorescence between individual cells in three species of algae. Each frequency of fluorescence intensity corresponds to the green fluorescent of individual cells in the bulk sample. We could expect that according to the intrinsic lipid content of single microalga, the lipid-rich microalgae can be classified and selected. Droplet-based microfluidics, which is capable of isolation of single microalga under the cell-friendly environment, would facilitate the quantitative estimation of the stochastic heterogeneity in algal lipid content.
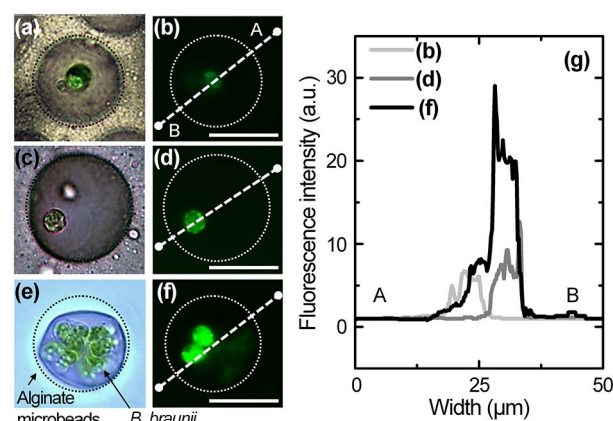

Figure 5: Enlarged bright-field and fluorescence images of the microbeads with single cells of (a,b) C. vulgaris, (c,d) Chlamydomonas sp., (e,f) B. braunii, respectively. Scale bars: 20 µm. (g) The fluorescence intensity profiles of each of the three microbeads across the dotted line AB.

We finally verified the lipid productivity of single algae cells among three algae species within the monodispersed microbeads. Figure 5a–f shows the bright-field and fluorescence images of the microbeads with single cells in three species of algae. This implies that the bright green fluorescence which represents the amount of intercellular lipids in an algal cell could be easily observed although the cell was encapsulated into the microbeads. The size of fabricated monodisperse alginate droplets with a mean diameter of 26 μm was enough to encapsulate single microalga, regardless of microalgal species. In addition, within the microbead, the difference of green fluorescence among three species was quantitatively estimated by direct observation using the fluorescence microscopy. We compared the fluorescence intensity profiles of each of the three microbeads across the dotted line AB (Figure 5g). As similar to Figure 3, *B. braunii* contains relatively high lipid content than *C. vulgaris* and *Chlamydomonas sp*.

CONCLUSIONS

In summary, we have massively fabricated the alginate hydrogel microbeads encapsulated single cells from three species of green microalgae (*Chlorella vulgaris*, *Chlamydomonas sp.* and *Botryococcus braunii*) using the microfluidic device incorporated microbridge structures. By utilizing the rapid migration of the additional control flow including calcified oil phase via the microbridge structures, we could stably fabricate the alginate hydrogel microbeads with the suitable spacing and prevent the aggregation of microbeads between their neighborhoods. The fabricated microbeads showed their own characteristics of monodispersity, robustness for enough handling, stability for the long-term manipulation, and possibility to maintain the cell viability. Also, combining the microbeads with the non-destructive fluorescence staining method with BODIPY, we could perform the quantitative interspecies comparison of the intracellular lipids and ensure the follow-up biological analysis. Similar to the literature data, there was a significant difference in the fluorescence intensity among three species within the hydrogel microbead due to the difference of the lipid content within the cells. The conservation of cell viability within the microbeads was also verified by the SYTOX Green staining. This implies that the microbead encapsulation was not affect to damage cells over a time period of the lipid screening. The proposed microbead-based platform for screening of lipid-rich microalgae is now technically ready-to-use for exploiting the alternative energy and will provide versatile tools for environmental engineering research.

ACKNOWLEDGEMENTS

REFERENCES

[1] J. Hill, E. Nelson, D. Tilman, S. Polasky, D. Tiffany, "Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels", *Proc. Natl. Acad. Sci. U.S.A.*, vol. 103, pp. 11206-11210, 2006.

[2] Y. Chisti, "Biodiesel from microalgae", *Biotechnol. Adv.*, vol. 25, pp. 294-306, 2007.

[3] R. H. Wijffels, M. J. Barbosa, "An outlook on microalgal biofuels", *Science*, vol. 329, pp. 796-799, 2010.

[4] M. J. Griffiths, R. P. van Hille, S. T. L. Harrison, "Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions", *J. Appl. Phycol.*, vol. 24, pp. 989-1001, 2012.

[5] S. Y. Teh, R. Lin, L. H. Hung, A. P. Lee, "Droplet microfluidics", *Lab Chip*, vol. 8, pp. 198-220, 2008.

[6] J. Pan, A. L. Stephenson, E. Kazamia, W. T. S. Huck, J. S. Dennis, A. G. Smith, C. Abell, "Quantitative tracking of the growth of individual algal cells in microdroplet compartments", *Integr. Biol.*, vol. 3, pp. 1043-1051, 2011.

[7] L. Mazutis, A. D. Griffiths, "Preparation of monodisperse emulsions by hydrodynamic size fractionation", *Appl. Phys. Lett.*, vol. 95, pp. 204103, 2009.

[8] E. Turmakin, E. Kumacheva, "Microfluidic generation of microgels from synthetic and natural polymers", *Chem. Soc. Rev.*, vol. 38, pp. 2161-2168, 2009.

[9] E. Um, D. S. Lee, H. B. Pyo, J. K. Park, "Continuous generation of hydrogel beads and encapsulation of biological materials using a microfluidic droplet-merging channel", *Microfluid. Nanofluid.*, vol. 5, pp. 541-549, 2008.

[10] D. H. Lee, W. Lee, E. Um, J. K. Park, "Microbridge structures for uniform interval control of flowing droplets in microfluidic networks", *Biomicrofluidics*, vol. 5, pp. 034117, 2011.

[11] E. Um, S. G. Lee, J. K. Park, "Random breakup of microdroplets for single-cell encapsulation", *Appl. Phys. Lett.*, vol. 97, pp. 153703, 2010.

[12] T. Govender, L. Ramanna, I. Rawat, F. Bux, "BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae", *Bioresour. Technol.*, vol. 114, pp. 507-511, 2012.

[13] H. Hwang, Y. J. Choi, W. Choi, S. H. Kim, J. Jang, J. K. Park, "Interactive manipulation of blood cells using a lens-integrated liquid crystal display based optoelectronic tweezers system", *Electrophoresis*, vol. 29, pp. 1203-1212, 2008.

CONTACT

* J.-K. Park, tel: 82-42-350-4315; jekyun@kaist.ac.kr