DNA hydrogel microspheres and their potential applications for protein delivery and live cell monitoring

Taeyoung Kim,¹ Seongmin Park,¹ Minhyuk Lee,¹ Solhee Baek,¹
Jong Bum Lee,² and Nokyoung Park^{1,a)}

¹Department of Chemistry, Myongji University, 116 Myongji-Ro, Cheoin-Gu, Yongin,
Gyeonggi-Do 449-728, South Korea

²Department of Chemical Engineering, University of Seoul, Seoul 130-743, South Korea

(Received 25 March 2016; accepted 18 May 2016; published online 26 May 2016)

Microfluidic devices have been extensively developed as methods for microscale materials fabrication. It has also been adopted for polymeric microsphere fabrication and *in situ* drug encapsulation. Here, we employed multi-inlet microfluidic channels for DNA hydrogel microsphere formation and *in situ* protein encapsulation. The release of encapsulated proteins from DNA hydrogels showed different profiles accordingly with the size of microspheres. *Published by AIP Publishing.*
[<http://dx.doi.org/10.1063/1.4953046>]

INTRODUCTION

Materials with the ability to entrap and subsequently release functional protein drugs have been widely studied and led to new treatments for a number of diseases.¹ Many synthetic polymers have been used to achieve long-term and controlled protein drug release.^{2–5} Recently, DNA hydrogels which are made entirely from DNA building blocks (X-, Y-, and T-DNA) have been developed.^{6,7} These DNA hydrogels were biocompatible, biodegradable, and inexpensive to fabricate. In addition, the efficient and ligase-mediated gelation process of DNA hydrogels provided several advantages over other bio-inspired polymers: (1) the gelling processes are achieved under physiological conditions and (2) drugs can be encapsulated *in situ* the gelation process eliminating the drug loading step. Based on these remarkable properties, it has been demonstrated that these DNA hydrogels can be used for *in-situ* protein drug encapsulation with high efficiency, and controlled insulin release up to 12 days from a bulk DNA hydrogel. Furthermore, DNA hydrogels showed the capability to entrap live cells and maintain the cell viability. Based on the results of the demonstrations, DNA hydrogels can be considered as promising scaffold materials which have great potential to be used for a protein drug delivery and a cell monitoring system. However, bulk gel has several limitations as (1) low accumulative release profile (lower than 60% of loaded proteins), (2) difficulty of injecting the gel for *in vivo* delivery, and precise drug release due to the large and irregular size of the hydrogels, and (3) it is not feasible to provide a confined environment for a single cell. To overcome these constraints of the bulk scale approach and improve the applicability of the DNA hydrogels, it is very important to develop methods which can produce an appropriate format of the hydrogel for various bio-applications.

Microfluidic devices have been extensively studied as methods for microscale materials fabrication. Especially, droplet generating devices had been employed to produce various types of natural hydrogel microdroplets which were successfully applied for drug deliveries and cell encapsulations.^{8–12} However, most of the microdroplet generations had been limited on protein- or polysaccharide-based hydrogel, and DNA hydrogels have not been studied in spite of their superior properties which were described above. Here, we report a method for fabrication of DNA hydrogel microspheres using a droplet generating microfluidic device to provide an

^{a)} Author to whom correspondence should be addressed. Electronic mail: pospnk@mju.ac.kr

appropriate microscale format of DNA hydrogels and their applications for efficient protein release and single cell monitoring.

MATERIALS AND METHODS

Microfluidic device fabrication

The microchannel was made by general microfabrication technique. In brief, this started from creating a channel pattern with computer-aided design software followed by transferring this pattern to a photomask using a pattern generator. The photomask was then used to make a SU-8 pattern on a silicon wafer. The UV exposure through the photomask and developing process left SU-8 pattern on the silicon wafer. This pattern was then imprinted onto PDMS through the crosslinking of liquid PDMS monomer. The channel pattern imprinted PDMS block was holed at the end of each channel for outer tubing connection. The PDMS block was cleaned by ultra sound sonication in ethanol and was bonded on to a PDMS coated glass slide resulting a microfluidic device. The width and depth of flow channels were 150 μm and 20 μm , respectively.

DNA hydrogel microsphere formation

The X-DNA building blocks were synthesized following the methods described in the previous reports.^{6,13,14} The X-DNA in nuclease free water and T4 DNA ligase in $2\times$ ligase buffer solution was introduced through inlet 1 and 2 separately by using syringe pump at the same flow rate. The mineral oil was introduced through inlet 3. The flow rate of each phase was controlled by syringe pumps. The X-DNA and the T4 ligase concentration in the droplet were 150 μM and 0.1 unit/ μl , respectively. The collected droplets were stored at room temperature for overnight for complete gelation.

Bovine serum albumin (BSA) encapsulation and release monitoring

BSA solution was prepared by dissolving BSA in nuclease free water and then mixed with DNA ligase solution to have the final concentration of 23 mg/ml in DNA micro-hydrogel. The collected micro-hydrogels were centrifuged down, and the oil was removed remaining minimum amount of oil. The protein release studies were conducted by suspending 30 μl of the collected micro-hydrogel of each size in 100 μl of $1\times$ PBS buffer (pH 7.4). The micro-hydrogel volumes were estimated from the flow rates of aqueous phase and collecting time periods, for example, the collecting for 10 min with flow rate of 0.1 μl per min gave 1 μl of hydrogel. The protein loaded hydrogel samples collected in $1\times$ PBS buffer were shaken at 70 revolutions per minute at room temperature. For every 24 h, the samples were centrifuged at 1500 rpm for 5 min to separate the hydrogels from the solution and 100 μl of the supernatant was removed. The samples were well mixed by short vortexing and returned to shaking incubation after addition of 100 μl of $1\times$ PBS blank solution. The removed supernatant solutions were used for measuring the released BSA amount using colorimetric Bradford assay by calibration with standards.

Cell encapsulation

HL-60 cells were stained with CellTrackerTM Red CMTPX (Invitrogen) and suspended in OPTi-MEM I media. The cell solution was mixed with DNA ligase solution to have the final concentration of 2570 cells/ μl in DNA micro-hydrogel. The X-DNA was stained with SYBR I to enhance the image contrast with encapsulated cells. The cells encapsulated in the micro-hydrogels were stored in 37 °C incubator between the microscopic imaging processes.

RESULTS AND DISCUSSION

Since the introduction of microfluidics based droplet formation method,¹⁵ it has been vastly employed to fabricate microspheres of diverse materials due to its ability to generate uniform-sized droplets and to control the sizes of microspheres. This method has been used to assemble

small colloidal particles into photonic balls¹⁶ and has also been used as a platform technology to monitor protein crystallization in a nanoliter droplet.¹⁷ In addition, protein drug release from monodisperse microspheres has been studied extensively due to their reproducibly controlled release behavior and the accessibility of alternative routes of administration including oral, pulmonary, subcutaneous, and intramuscular pathways.^{18–21} In this study, a microfluidic device as shown in Figure 1(a), composing of three inlets (two aqueous phase and one oil phase), a flow channel, and an outlet has been used to generate uniform size of aqueous microdroplets functioning as a DNA ligation microreactor to crosslink X-DNA into hydrogel. Syringe pumps were used to introduce the liquid components into the inlet channels and to control the flow rates of each phase. As shown in Figure 1(a), the X-DNA and the DNA ligase were injected separately through inlets 1 and 2 to prevent an early gelation which prohibits formation of uniform microdroplets, merging in a short mixing channel prior to the junction. The mixture containing X-DNA and DNA ligase was then sheared at the junction by an oil flow injected through inlet 3 generating microdroplets (Figure 1(b)) kept the droplets in the oil phase.

The sizes of micro-hydrogel droplets (20–90 μm in diameter) were controlled by altering the ratio of flow rates between aqueous phase and oil phase (Figures 2(a) and 2(b)), and the collected hydrogel microspheres had reasonably uniform size according to the measuring from the microscopic images. For example, the size of micro-hydrogel produced at the flow rate ratio of 0.12 (aqueous/oil) was measured to $21.9 \pm 2.6 \mu\text{m}$ (Figures 2(c) and 2(d)). To confirm the gelation of X-DNA in a microdroplet which has approximately 4 μl volume, microdroplets collected in oil phase (Figure 2(c)) were transferred to aqueous phase by repeated steps of centrifugation, removal of oil supernatant, and suspension of the droplets into nuclease free water. After transferring into water, the DNA micro-hydrogel was stained with SYBR I, which is a double stranded DNA-specific green fluorescent dye and observed under a microscope. The defined green fluorescence spheres confirmed the formation of DNA micro-hydrogels with uniform sizes of $20.5 \pm 3.2 \mu\text{m}$ (Figures 2(c) (inset) and 2(d)) and also successful enzymatic ligation in μl scale microdroplets.

To investigate the potential of the DNA hydrogel microsphere as a protein drug carrier, the controlled release of bovine serum albumin (BSA) from different size of DNA hydrogel microspheres was monitored. BSA was encapsulated in micro-hydrogel by introducing a BSA and DNA ligase mixture from a single channel (inlet 2) separately from X-DNA channel (inlet 1). In this study, two different sizes (23.3 and 32.1 μm in diameter) of BSA encapsulating micro-hydrogel were used for a protein release profiling. The BSA release from DNA hydrogel microspheres was monitored for 14 days as shown in Figure 3. The entrapped BSA was linearly released without bursting and the release from the DNA micro-hydrogel was size dependent. These release profiles were well correlated with previous report studied with bulk DNA

FIG. 1. (a) A schematic drawing of microchannels. (b) A micro-scopic image of channels and droplets being generated.

FIG. 2. (a) DNA hydrogel droplets generation at different flow rates of inlet 1, 2, and 3 (flow rates are normalized to that of inlet 3). (b) Droplet sizes at the different ratios of aqueous and oil phase flow rates (open diamond: oil flow fixed and aqueous flow rates are varied, closed circle: aqueous flow fixed and oil flow rates are varied. aqueous phase rates are calculated by combining flow rates of inlet 1 and inlet 2). (c) DNA hydrogel droplets collected at the ratio of 0.12 in oil phase (inset: transferred DNA hydrogel microspheres to water and stained with SYBR I). (d) Size distributions of DNA hydrogels (black bars: in oil phase, gray bars: after transferred to aqueous phase).

hydrogels presenting that DNA hydrogel is biodegradable and the encapsulated proteins were released from DNA hydrogel by the degradation.⁶ The BSA release from 23.3 and 32.1 μm hydrogel reached saturation point in 5 and 7 days, respectively, and the reason of the difference could be explained as the time needed for hydrogel degradation which is necessary for BSA

FIG. 3. BSA release profiles from two different sizes of DNA hydrogel microspheres. Open diamond and closed square indicate 23.3 and 32.1 μm of hydrogel microsphere diameter, respectively.

FIG. 4. (a) Fluorescence image of the microchannel junction where DNA and cell solutions, stained with green and red fluorescent dyes, respectively, merged. (b) and (c) Images of a single cell encapsulating into a DNA hydrogel microdroplet. (d) Fluorescence image of cells encapsulated within DNA hydrogel microspheres.

release is longer for the larger microsphere than smaller one. The accumulative quantity of released proteins was close to 100% of that of loaded proteins for both of the droplet sizes. These results show that the DNA micro-hydrogel demonstrated the ability of controlled release for proteins.

To explore more applications of DNA micro-hydrogel, we studied its ability to encapsulate a live single cell. Although single cell behavior in a microenvironment has been studied extensively by encapsulating individual cell into a droplet,^{22,23} most of the researches had been performed in solution phase droplets which are not easy to replicate the extracellular matrix environment which is very dense. In this study, we have employed DNA micro-hydrogel which provides interconnected and very dense environment to develop a high-throughput single cell monitoring system. The physiological conditions of DNA gelation process allowed premixing the cells with gel precursor and kept the cells alive through the entire process of micro-hydrogel formation. Live cells were encapsulated into the hydrogel microsphere using the similar process of the protein encapsulation. Leukaemia (HL-60) cells suspended in a cell media were mixed with ligase solution and were introduced through one inlet to be mixed with X-DNA building blocks prior to the junction (Figures 4(a)–4(c)). The cells entrapped in the generated droplets were encapsulated into DNA micro-hydrogel by the ligation process. A cell dye which emits red fluorescence only in live cells was used to confirm the cell survival within the DNA micro-hydrogel. As shown in Figure 4(d), the cells in DNA micro-hydrogel emitted red fluorescence suggesting the cell viability in the DNA micro-hydrogel. According to a cell survival assay in a DNA hydrogel, the HL-60 cells did survive for 7 days without exchanging media. With the ability of encapsulating the individual live cell in a micro-hydrogel and keeping them alive for several days, this method has a potential to be a platform technology for a single cell monitoring system under physiological condition.

CONCLUSIONS

In summary, we present a new method to produce DNA micro-hydrogel and its applications for protein delivery and single cell encapsulation. The micro-hydrogels have been fabricated using microfluidic droplet generation and enzyme catalyzed ligation. The size of micro-hydrogel could be altered to have different controlled protein release profiles. Furthermore, it

has also been used to encapsulate individual live cell and monitor it for several days in a micro-hydrogel. Since each micro-hydrogel volume is extremely small (pl scale), the cost of monitoring will be much cheaper than conventional systems using microtiter plate. More importantly, because the cells were encapsulated within a hydrogel which may provide extracellular matrix mimic environment, it will be possible to monitor cell's behavior more similar to *in-vivo* situation. This DNA micro-hydrogel should open a new way of a high-throughput single cell monitoring system, providing unique hydrogel microenvironment as well as a protein controlled release system.

ACKNOWLEDGMENTS

This work was supported by 2015 Research Fund of Myongji University.

- ¹K. Fu, A. M. Klibanov, and R. Langer, *Nat. Biotechnol.* **18**, 24 (2000).
- ²R. Langer and J. Folkman, *Nature* **263**, 797 (1976).
- ³R. Langer, *Science* **249**, 1527 (1990).
- ⁴O. L. Johnson, J. L. Cleland, H. J. Lee, M. Charnis, E. Duenas, W. Jaworowicz, D. Shepard, A. Shahzamani, A. J. S. Jones, and S. D. Putney, *Nat. Med.* **2**, 795 (1996).
- ⁵G. Z. Zhu, S. R. Mallery, and S. P. Schwendeman, *Nat. Biotechnol.* **18**, 52 (2000).
- ⁶S. H. Um, J. B. Lee, N. Park, S. Y. Kwon, C. C. Umbach, and D. Luo, *Nat. Mater.* **5**, 797 (2006).
- ⁷N. Park, S. H. Um, H. Funabashi, J. F. Xu, and D. Luo, *Nat. Mater.* **8**, 432 (2009).
- ⁸M. Lian, C. P. Collier, M. J. Doktycz, and S. T. Retterer, *Biomicrofluidics* **6**, 044108 (2012).
- ⁹J. Wan, *Polymers* **4**, 1084–1108 (2012).
- ¹⁰S. Sugaya, M. Yamada, A. Hori, and M. Seki, *Biomicrofluidics* **7**, 054120 (2013).
- ¹¹L. Yu, S. M. Grist, S. S. Nasser, E. Cheng, Y.-C. E. Hwang, C. Ni, and K. C. Cheung, *Biomicrofluidics* **9**, 024118 (2015).
- ¹²C. Martino, T. Y. Lee, S.-H. Kim, and A. J. deMello, *Biomicrofluidics* **9**, 024101 (2015).
- ¹³Y. G. Li, Y. D. Tseng, S. Y. Kwon, L. D'Espaux, J. S. Bunch, P. L. Mceuen, and D. Luo, *Nat. Mater.* **3**, 38 (2004).
- ¹⁴S. H. Um, J. B. Lee, S. Y. Kwon, Y. Li, and D. Luo, *Nat. Protoc.* **1**, 995 (2006).
- ¹⁵T. Thorsen, R. W. Roberts, F. H. Arnold, and S. R. Quake, *Phys. Rev. Lett.* **86**, 4163 (2001).
- ¹⁶G. R. Yi, S. J. Jeon, T. Thorsen, V. N. Manoharan, S. R. Quake, D. J. Pine, and S. M. Yang, *Synth. Met.* **139**, 803 (2003).
- ¹⁷B. Zheng, J. D. Tice, L. S. Roach, and R. F. Ismagilov, *Angew. Chem., Int. Ed.* **43**, 2508 (2004).
- ¹⁸C. Berkland, K. K. Kim, and D. W. Pack, *J. Controlled Release* **73**, 59 (2001).
- ¹⁹N. Murthy, Y. X. Thng, S. Schuck, M. C. Xu, and J. M. J. Frechet, *J. Am. Chem. Soc.* **124**, 12398 (2002).
- ²⁰L. Y. Wang, G. H. Ma, and Z. G. Su, *J. Controlled Release* **106**, 62 (2005).
- ²¹H. G. Zhu and M. J. McShane, *Chem. Commun.* **2006**, 153.
- ²²J. Clausell-Tormos, D. Lieber, J. C. Baret, A. El-Harrak, O. J. Miller, L. Frenz, J. Blouwolff, K. J. Humphry, S. Koster, H. Duan, C. Holtze, D. A. Weitz, A. D. Griffiths, and C. A. Merten, *Chem. Biol.* **15**, 875 (2008).
- ²³E. Brouzes, M. Medkova, N. Savenelli, D. Marran, M. Twardowski, J. B. Hutchison, J. M. Rothberg, D. R. Link, N. Perrimon, and M. L. Samuels, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14195 (2009).