Macromolecular Materials and Engineering
www.mme-journal.de

Water-Triggered Rapid Release of Biocide with Enhanced Antimicrobial Activity in Biodiesel

*Hao Pei, Alireza Abbaspourrad, Weixia Zhang, Zhengwei Wu, and David A. Weitz**

Biodiesel inherently contains more water than mineral diesel and as a result microbial contamination is a major problem that hinders its widespread application. The current method of removing the microbial contamination is direct addition of biocide. However, this method cannot enrich the water phase with biocide rapidly enough, leading to unavoidable overdosing of biocide and environmental issues. Here, biocide is encapsulated within hydrogel microparticles with a water-triggered release feature to improve antimicrobial efficiency of biocide in biodiesel. To demonstrate the water-triggered release mechanism, a green dye is encapsulated within the microparticles. The encapsulated dye remains inside the microparticles for more than 6 weeks when the microparticles are stored in oil phase; however, the dye releases in 4 min when the microparticles contact water. Using this water-triggered release strategy, biocide is successfully delivered to the water phase in biodiesel. The encapsulated biocide shows higher antimicrobial efficacy than that of free biocide, in both short-term and long-term experiments. The possibility of scaling up the production of hydrogel microparticles using bulk emulsification method is also explored. Moreover, the water-triggered release strategy can be used for releasing other water-soluble functional materials. This opens opportunities for a wide range of encapsulation and controlled delivery applications.

Biodiesel, an alternative diesel fuel that is produced from renewable biological resources such as vegetable oils and animal fats, is becoming more attractive due to its environmental benefits and economic feasibility.[1–3] This renewable diesel is very similar to petroleum diesel in many properties; hence, it can be blended with mineral diesel in any proportion and can be used to power diesel engines without any engine modifications.[4] A major problem that hinders wide application of biodiesel is microbial contamination during biodiesel storage and distribution.[5] Biodiesel has a higher quantity of nutrients than mineral diesel, providing microorganisms with abundant food. In addition, biodiesel absorbs 10–15 times more water than mineral diesel at the same temperature, resulting in free water settling at the bottom of the tank or finely distributed in the biodiesel.[5,6] Together with water formed from condensation due to temperature fluctuations, this free water provides an ideal environment for microbial community growth. Microorganisms are ubiquitous, existing in the soil, air, animals, as well as in biodiesel. The growth of microorganisms such as bacteria, fungi, and yeasts starts at the oil–water interface and then expands into the aqueous phase.[7,8] The microbial growth results in decline in product quality, formation of biomass and bio-sludge, and pipe and filter blockage. Moreover, the acidic products in microbial metabolism will corrode the storage tank and fuel injection system.[9–11] Additionally, the microbial contamination can be passed on from refinery to storage tank to end-uses.

Several methods have been developed to inhibit or even prevent microbial contamination in biodiesel.[12–15] One approach is to remove the free water from biodiesel, including water deposits and emulsified water, which makes the microbes dormant and prevents microbe proliferation.[12–14] In this approach, a superhydrophobic filter is utilized to block water and allow the oil phase to easily pass, thus separating water and oil. However, high cost and low reusability of the filter media limits this approach. Another method uses anti-corrosive and antimicrobial coatings in the fuel tank,[15] but the coating is not durable and can deteriorate over time when exposed to some extracellular microbial products. A more widely applied method is direct addition of an antimicrobial active or biocide into the fuel to kill the microbes once the fuel is contaminated.[9,16–18] In this approach, the biocide molecules diffuse from the fuel into the free water in the biodiesel, where the microbes live and multiply. However, the biocide diffusion rate is slow and depends on many environmental factors such as temperature, vibration, and tank geometry. Thus, this approach cannot enrich the water phase with biocides rapidly enough to kill the microbes in short

Dr. H. Pei, Dr. W. Zhang, Z. Wu, Prof. D. A. Weitz
John A. Paulson School of Engineering and Applied Sciences
Harvard University
Cambridge, MA 02138, USA
E-mail: weitz@seas.harvard.edu
Prof. A. Abbaspourrad
Department of Food Science
Cornell University
Ithaca, NY 14853, USA
Z. Wu
Department of Biomedical Engineering and Biotechnology
University of Massachusetts Lowell
Lowell, MA 01854, USA
The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mame.201900156.

DOI: 10.1002/mame.201900156

periods of time. To expedite the diffusion process, a large quantity of biocide is added to the fuel in practice, leading to unavoidable overdosing of biocide. The remaining biocide in the fuel eventually burns with biodiesel, resulting in low efficiency of usage of the antimicrobial actives as well as increased air pollution. More importantly, the remaining biocide in storage tanks will cause severe safety issues due to the toxicity of the biocide.[19–22] Therefore, there is a need for smarter and more efficient delivery of biocide that targets water phase directly using smaller doses of biocide added to fuel to enhance biocide efficiency and to reduce the toxic risk arising from the remaining biocide.

In this paper, we present a strategy for encapsulation and targeted delivery of biocide to the water phase in biodiesel. The microparticle is designed for water-triggered release. The microparticles are made of hydrogel; the cargo is kept in the matrix of the microparticles when in oil; upon contact with water, the microparticles absorb water and the hydrogel network swells, releasing the cargo. We fabricate a water-in-oil single emulsion that contains hydrophilic photocurable polymer and model biocide in the droplets using polydimethylsiloxane (PDMS) microfluidic devices, which are widely used to enable exquisite control on particles sizes, providing monodisperse emulsions for systematic studies.[23–33] The droplets are then photopolymerized with UV radiation to obtain hydrogel microparticles. After addition of these hydrogel microparticles to the fuel, they sediment to the interface of oil and water and then rapidly swell and release the biocide into the aqueous phase. We also perform bulk emulsification by using a homogenizer to enable this strategy to be applied for mass production. To evaluate the biocidal efficacy of the microparticles, we conduct antimicrobial activity experiments. The results demonstrate both short-term and long-term enhanced biocidal efficacy of microparticles in biodiesel.

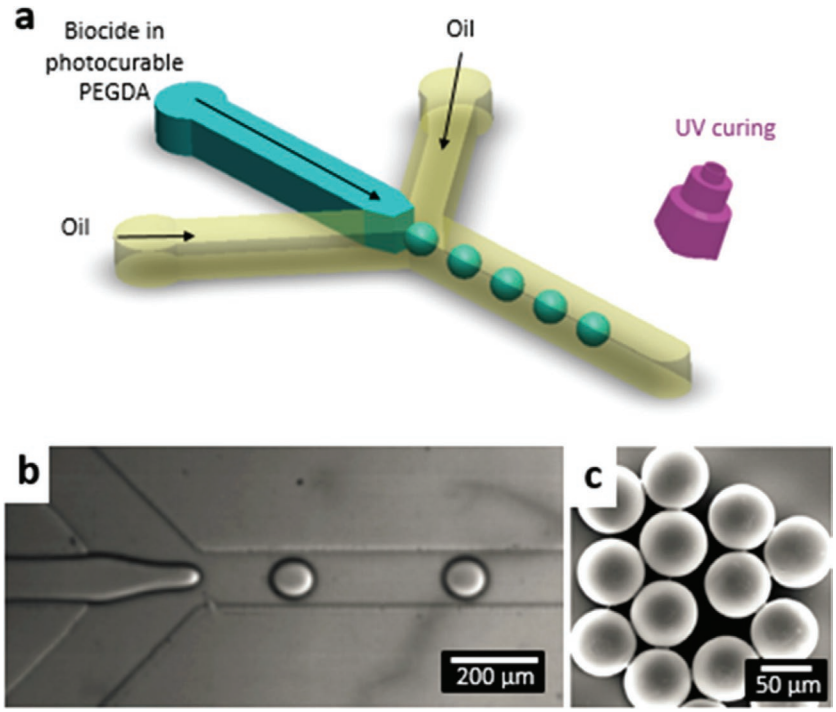

Figure 1. a) Schematic illustration of the poly (dimethylsiloxane) microfluidic device for preparing polymer microparticles encapsulating a popular biocide Grotomer71. b) Optical microscopic image shows the generation of single emulation drops containing biocide with a PDMS device. The photocurable hydrophilic PEGDA in the droplets polymerizes to form hydrogel network upon UV irradiation. c) Scanning electron microscopic image shows the hydrogel monodispersed microparticles.

We use a PDMS microfluidic device to prepare microparticles containing biocide (Experimental Section, Supporting Information). The devices are fabricated using soft-lithography.[26] To increase the hydrophobicity of the channels, we applied Aquapel to flush all the channels in the device. The PDMS device has two inlets and one outlet. A schematic of the device is illustrated in **Figure 1**a.

A model commercial biocide, Grotamar71, is chosen for its efficient biocidal activity and high water solubility. We use this PDMS microfluidic device to emulsify the water phase containing biocide in the oil phase. The aqueous phase is prepared by dissolving 4 wt% biocide, 40 wt% photocurable hydrophilic poly(ethylene glycol) diacrylate (PEGDA), 1 wt% photoinitiator, and a trace of fluorescein sodium salt in DI water. We inject this aqueous solution into the device through polyethylene tubing connected to the first inlet of the PDMS microfluidic device. An oil phase consisting of 98 wt% dodecane and 2 wt% surfactant EM 90 is injected into the device simultaneously with the outer phase through the second inlet to produce monodisperse single emulsion water-in-oil drops. Using this technique, we encapsulate the biocide in the aqueous phase droplets; a microscopic image of droplet formation within the operating device is shown in Figure 1b. Following droplet generation, UV irradiation is applied at the exit of the polyethylene tubing to polymerize the photocurable hydrophilic PEGDA. The resultant hydrogel microparticles containing biocide are collected in the oil phase. The monodisperse hydrogel microparticles are shown in Figure 1c.

To visualize the cargo release process of the hydrogel microparticles upon exposure to water, we encapsulate a green fluorescent dye (fluorescein sodium salt) in the disperse phase during the droplet generation and thereafter use confocal microscope to directly examine the cargo release. The hydrogel microparticles containing dye are first dispersed in oil. Subsequently, water is slowly introduced into the oil dispersion of the hydrogel microparticles. Due to the higher density of water than oil, water accumulates at the bottom of the vial.

Upon contact with water, the fluorescent dye leaks out immediately, as evidenced by the expansion of the green area as shown in time lapse confocal images in **Figure 2**a. By comparison, the release of the fluorescent dye is negligible in oil and the fluorescent hydrogel microparticles can maintain their shapes for more than 6 weeks. We attribute this water-triggered release to the hydrophilic nature of the hydrogel and expansion of the network of the microparticles upon contact with water.

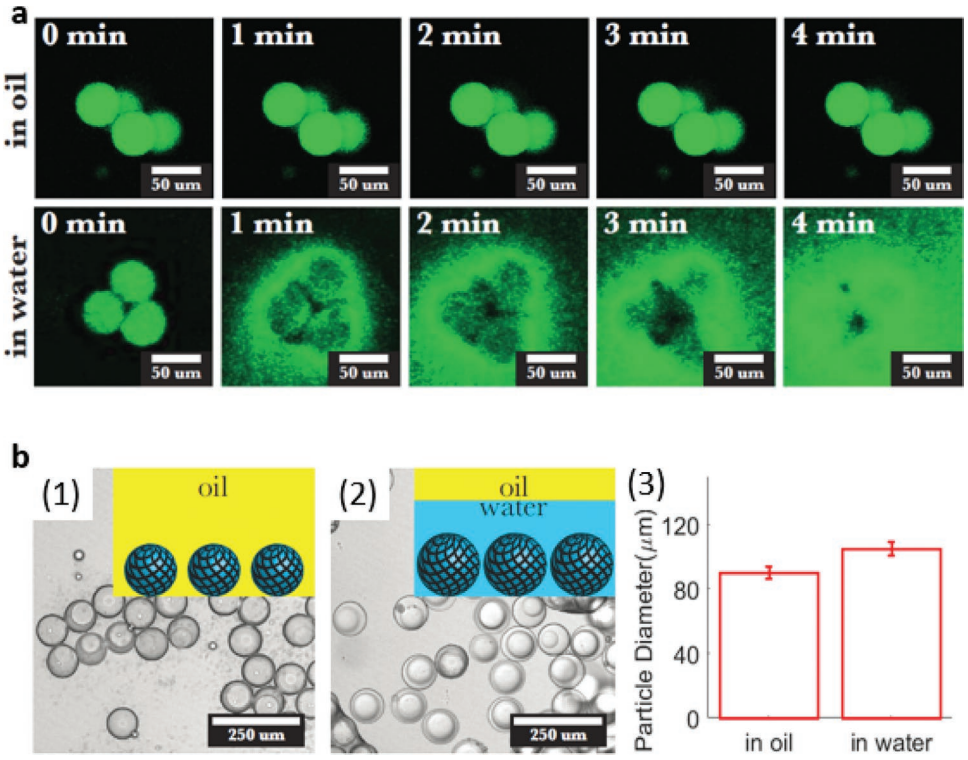

Figure 2. a) Fluorescence microscopic images of fluorescein-encapsulated hydrogel microparticles in oil and in water after exposure to water for different time lengths. The green color is from the fluorescein dye. b) Microscopic images of Grotamar71-encapsulated hydrogel microparticles in oil and in water. The yellow color in the schematic illustration represents oil whereas the blue color represents water. 1) Hydrogel microparticles are collected with continuous phase. 2) As soon as water is added into the dispersion, hydrogel microparticles swell and the water permeability is therefore increased as a result of the formation of a loose structure. 3) Graph showing the diameter of the microparticles in oil phase and the diameter of the same microparticles in water.

To quantify the water-triggered swelling of the hydrogel microparticles, we measure and compare the size before and after exposure to water. The hydrogel microparticles containing biocide are first dispersed in dodecane as shown by the microscopic picture. Since the microparticles are denser than the oil phase, they all sediment to the bottom of the vial, as shown in Figure 2b(2). Then, we slowly add water to the oil dispersion of the hydrogel microparticles. Water fills the bottom of the container and starts to contact with hydrogel microparticles. It quickly wets the microparticles and penetrates into the microparticles, causing them to swell, as shown in Figure 2b(2). The average size of the hydrogel microparticles increase from 90.1 to 105.1 μm, increasing the volume by approximately 40%, as shown in the bar chart in Figure 2b(3). Therefore, upon exposure to water and the subsequent swelling of the hydrogel microparticles, the hydrophilic cargo is released into the water phase.

To investigate the antimicrobial efficacy of free and encapsulated biocide, we perform an antimicrobial activity experiment. We choose *Bacillus subtilis* as our microorganism model and use Grotamar71 as the model biocide; this is an effective biocide widely used in diesel fuel that kills a broad spectrum of aerobic and anaerobic microorganisms such as fungi, bacteria, and yeast.[34] We prepare a biodiesel solution of free biocide and a biodiesel suspension of encapsulated biocide with the same biocide concentration ranging from 100 to 400 μg L^{-1}. Then, we add aqueous bacteria broth into the biodiesel and let it stand overnight to enable the microparticles to absorb water and then to be transported into the water phase to release the encapsulated biocide; because water and oil are immiscible, they will phase separate. The oil phase with smaller density will be the supernate. We then remove the oil phase from the aqueous phase. We inoculate *B. subtilis* in as-prepared aqueous broth and incubate *B. subtilis* in as-prepared aqueous broth and measure the bacteria density after 3, 6, 12, 24, and 72 h incubation.

After 3 h incubation with free biocide, we observe a decrease in bacterial viability from approximately 85% to 20%, as the concentration of biocide increases from 100 to 400 μg L^{-1}, as shown in **Figure 3**a. When the incubation time is extended to 6, 12, and 24 h, a similar decrease in bacteria viability is obtained but the decrease becomes less and less pronounced, as shown in Figure 3b–d. However, the viability of *B. subtilis* is diminished significantly after encapsulating the biocide within the hydrogel microparticles. Moreover, we observe that the viability of the bacteria for low concentration of biocide (100, 150, and 200 μg L^{-1}) before encapsulation increases as time goes, while the viability decreases for all concentrations of encapsulated biocide. In the case of 200 μg L^{-1} of biocide, the bacterial viability increases from 37% to 94% as the incubation time goes from 3 to 24 h; by contrast, the bacterial viability decreases from 19% to 3% for the same concentration of encapsulated biocide during the same incubation period.

The significant decrease in bacterial viability after encapsulation demonstrates the higher antimicrobial efficiency

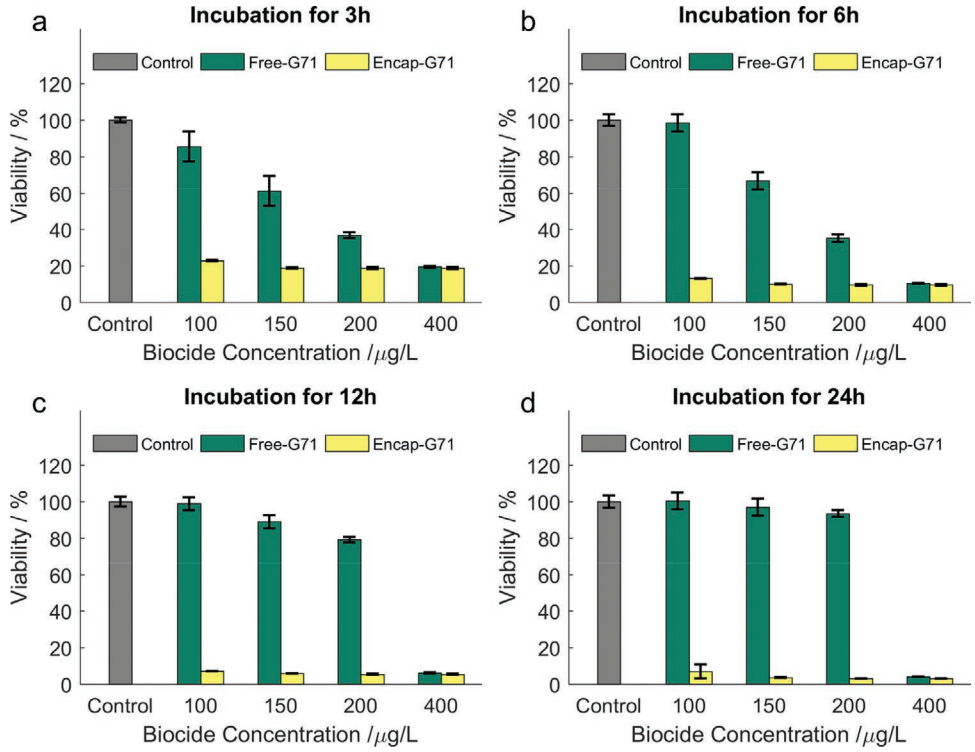

Figure 3. Viability of *Bacillus subtilis* after a) 3, b) 6, c) 12, and d) 24 h incubation upon treatment of different concentrations (μg L^{-1}) of the biocide assessed by 600 wavelength optical density meter. Free-G71 is for free biocide; Encap-G71 is for encapsulated biocide. All the experiments are conducted at 30 °C. Error bars represent the mean ± SD ($n = 3$).

of the hydrogel microparticles. We attribute the enhanced antimicrobial activity to rapid release of biocide and immediate high concentration of biocide in the aqueous broth. Although the biodiesel solution of biocide and biodiesel suspension of encapsulated biocide have the same concentrations of biocide, expressed in units of micrograms of biocide per liter of biodiesel, biocide molecules go to the aqueous phase much faster in the suspension. The burst-release rate of the biocide in the suspension is far higher than the diffusion rate of the biocide in the solution from the oil to the broth, resulting immediate higher concentration of biocide in the broth. The increased bacterial viability at low concentrations of free biocide (100, 150, and 200 μg L^{-1}) over time is due to insufficient amounts of biocide. After 3 h incubation, the bacterial viability decreases significantly, but the viability starts to increase after 6, 12, and 24 h of incubation, since the remaining biocide is not enough to maintain the bacterial viability at a low level and the bacteria start to multiply again. By contrast, for 400 μg L^{-1} of free biocide, the bacterial viability continues to decrease after 3, 6, 12, and 24 h of incubation, which demonstrates that a sufficient biocide is present in the broth.

The antimicrobial activity is further evaluated through determination of the minimum inhibitory concentration (MIC). The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the visible growth of a microorganism for overnight incubation. The growth of bacteria is completely inhibited upon treatment of 400 μg L^{-1} biocide, while after encapsulation, 100 μg L^{-1} biocide is sufficient to inhibit the growth of *B. subtilis*, as shown in Figure 3c. These MIC values show an enhanced antimicrobial efficacy for short term.

To examine the long-term persistence of maintaining the bacteria at low concentrations, a 72-h incubation is conducted. More than 99.6% of the bacteria are killed after 72 h

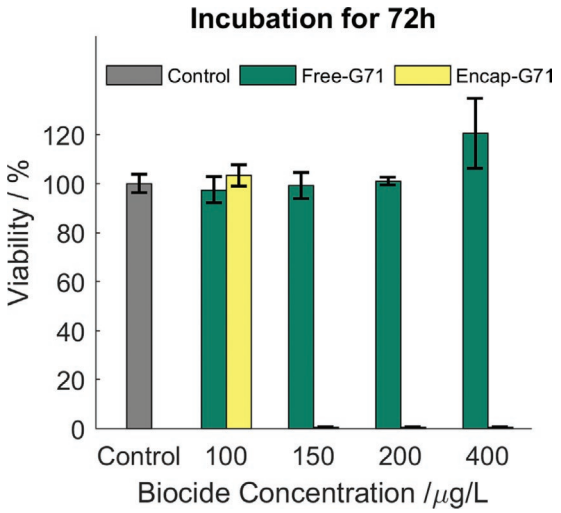

Figure 4. Viability of *Bacillus subtilis* after 72 h incubation on exposure to different doses of biocide assessed by a 600 nm optical density meter. All the experiments are conducted at 30 °C. Error bars represent the mean ± SD ($n = 3$).

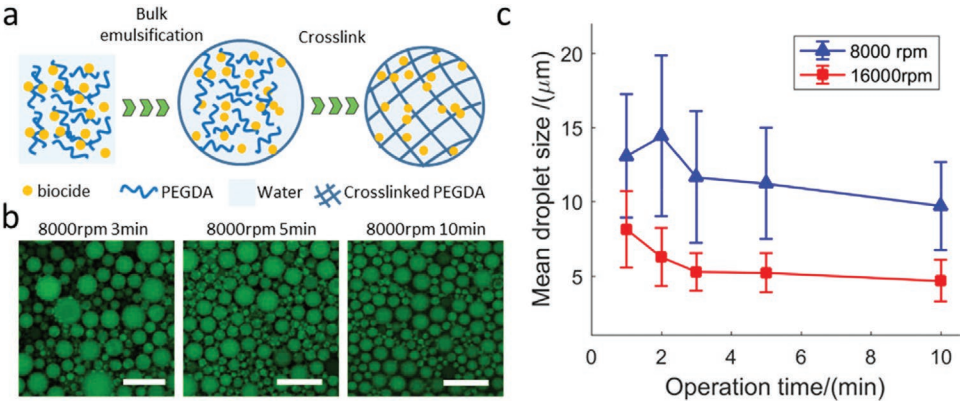

Figure 5. Bulk emulsification for mass production. a) The schematic figure showing procedure of mass producing hydrogel microparticles containing biocide by using bulk emulsification. b) Graph showing the mean size of the hydrogel microparticles versus operation time with different operating speeds. c) Fluorescence microscopic images of hydrogel microparticles emulsified for various times, from 3 to 10 min. Scale bar: 50 μm.

of incubation upon treatment of the 150, 200, and 400 μg L^{-1} encapsulated biocide broth, whereas the bacterial viability recovers to approximately 100% at all concentrations of free biocide broth, as shown in **Figure 4**. This demonstrates that the encapsulation has good potential in preserving biodiesel against microorganisms for long periods of time with less dose of the biocide.

We use microfluidic device to produce monodisperse drops with tunable sizes, providing a firm basis for systematic study. However, this limits throughput to milliliters per hour or less.[35–38] To facilitate the potential mass production of the hydrogel microparticles containing biocide, a homogenizer is used to bulk emulsify the aqueous disperse phase containing biocide and photocurable hydrophilic PEGDA in the continuous phase of dodecane and 2 wt% of EM 90 as the surfactant. Subsequently, we use a high-power UV lamp (EXFO S1000 OminiCure Platform) to solidify the dispersed phase. Upon exposure to UV light, the polymer is crosslinked and the network is formed. The micro-structure of the microparticles and the procedure for mass production with the homogenizer are shown in **Figure 5**a. The hydrogel particles are then thoroughly washed with pure dodecane to remove the residual surfactant in the oil phase.

To control the particle size distribution, we make the emulsions with a homogenizer using different speeds and operation times. Fluorescein sodium salt is added to the dispersed phase to directly visualize the emulsion droplets in the oil, as shown in Figure 5b. By image processing of the fluorescence microscopic images, we determine how the two key factors, rotation speed and operation time, influence the hydrogel microparticle mean size. We observe a decrease in the mean size of the hydrogel microparticles, from approximately 13 to 9 μm, as the homogenization time increases from 1 to 10 min when the rotation speed is 8000 rpm, as shown in Figure 5c. Similarly, we observe a decrease in the mean size of the hydrogel microparticles as a function of homogenization time, when the speed is 16 000 rpm. Moreover, a longer homogenization time leads to a smaller standard deviation in the particle size, resulting in more uniform microparticles. We also observe that the mean size is much smaller when high rotation speed is used. In the case of 10 min of homogenization time, the mean sizes are approximately 9 and 4 μm for 8000 and 16 000 rpm, respectively. Hence, the homogenizer can control both the mean size and the standard deviation of the hydrogel microparticles.

In this work, we encapsulate biocide within hydrogel microparticles with a water-triggered release feature to improve antimicrobial efficacy of biocide in biodiesel. To demonstrate the water-triggered release mechanism, we encapsulate a green dye as a model within microparticles. When the microparticles are stored in oil phase, we find the dye remains encapsulated for more than 6 weeks. In contrast, all the dye releases in 4 min after exposing the microparticles to water. We attribute this water-triggered release to the hydrophilic nature of the hydrogel and expansion of the network of the microparticles upon contact with water. Using this water-triggered release strategy, we successfully deliver biocide to the water phase in biodiesel. This targeted delivery of biocide shows higher antimicrobial efficacy than that of free biocide, in both short-term and long-term experiments. We also explore the possibility of scaling up the production of hydrogel microparticles using bulk emulsification method. Moreover, the water-triggered release strategy can be used for releasing other water-soluble functional materials. This opens new opportunities for a wide range of encapsulation and controlled delivery applications.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by Total (A27745), the National Science Foundation (DMR-1310266), and the Harvard Materials Research Science and Engineering Center (DMR-1420570).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

biocides, biodiesel, enhanced antimicrobial activity, microgels, water-triggered release

Received: March 14, 2019
Revised: May 7, 2019
Published online:

[1] G. Knothe, J. Krahl, J. Van Gerpen, *The Biodiesel Handbook*, Elsevier, Amsterdam **2015**.
[2] F. Ma, M. A. Hanna, *Bioresour. Technol.* **1999**, *70*, 1.
[3] W. Wang, D. Lyons, N. Clark, M. Gautam, P. Norton, *Environ. Sci. Technol.* **2000**, *34*, 933.
[4] A. K. Agarwal, *Prog. Energy Combust. Sci.* **2007**, *33*, 233.
[5] M. D. Vangsness, L. Bowen, L. Brown, S. Mueller, L. Balster, in *The 31st Symp. on Biotechnology for Fuels and Chemicals*, Society for Industrial Microbiology, San Francisco, CA **2009**.
[6] P. B. L. Fregolente, L. V. Fregolente, M. R. Wolf Maciel, *J. Chem. Eng. Data*, **2012**, *57*, 1817.
[7] W. Siegert, in *Proc. of the 11th Int. Conf. on the Stability and Handling of Liquid Fuels* (Ed: R. E. Morris), IASH, Atlanta, GA **2009**, pp. 13531412.
[8] B. Leuchtle, W. Xie, T. Zambanini, S. Eiden, W. Koch, K. Lucka, M. Zimmermann, L. Blank, *Energy Fuels* **2015**, *29*, 6394.
[9] F. Passman, *Int. Biodeterior. Biodegrad.* **2013**, *81*, 88.
[10] F. Bücker, N. Santestevan, L. Roesch, R. Jacques, M. Peralba, F. Camargo, F. Bento, *Int. Biodeterior. Biodegrad.* **2011**, *65*, 172.
[11] C. Gaylarde, F. Bento, J. Kelley, *Rev. Microbiol.* **1999**, *30*, 01.
[12] S. Patel, G. Chase, *Sep. Purif. Technol.* **2014**, *126*, 62.
[13] J. Zhao, W. Ren, H-M. Cheng, *J. Mater. Chem.* **2012**, *22*, 20197.
[14] G. Viswanadam, G. Chase, *Sep. Purif. Technol.* **2013**, *104*, 81.
[15] E. Hill, G. Hill, *Advanced Materials Research,* Trans Tech Publications, Switzerland **2008**.
[16] W. Siegert, P. Lee. in *SPE Int. Conf. on Health, Safety and Environment in Oil and Gas Exploration and Production*, Society of Petroleum Engineers, Kuala Lumpur, Malaysia **2002**.
[17] A. Zimmer, J. Cazarolli, R. Teixeira, S. Viscardi, E. Cavalcanti, A. Gerbase, M. Ferrao, C. Piatnicki, F. Bento, *Fuel* **2013**, *112*, 153.
[18] J. Robbins, R. Levy, in *Directory of Microbicides for the Protection of Materials* (Ed: W . Paulus), Springer, Berlin **2004**, p. 177.
[19] G. Luz, B. Sousa, A. Guedes, C. Barreto, L. Brasil, *Molecules* **2018**, *23*, 2698.
[20] R. Poon, M. Rigden, N. Edmonds, N. Charman, S. Lamy, *Arch. Toxicol.* **2011**, *85*, 1419.
[21] S. Banga, P. Varsheny, *J. Sci. Ind. Res.* **2010**, *69*, 575.
[22] N. Yemashova, V. Murygina, D. Zhukov, A. Zakharyantz, M. Gladchenko, V. Appanna, S. Kalyuzhnyi, *Rev. Environ. Sci. Bio/Technol.* **2007**, *6*, 315.
[23] A. Abate, D. Weitz, *Small* **2009**, *5*, 2030.
[24] W. Duncanson, T. Lin, A. Abate, S. Serffert, R. Shah, D. Weitz, *Lab Chip*, **2012**, *12*, 2135.
[25] R. Shah, H. Shum, A. Rowat, D. Lee, J. Agresti, A. Utada, L. Chu, J. Kim, A. Fernandez-Nieves, C. Martinez, D. Weitz, *Mater. Today* **2008**, *11*, 18.
[26] H. Lee, C. Choi, A. Abbaspourrad, C. Wesner, M. Caggioni, T. Zhu, D. Weitz, *ACS Appl. Mater. Interfaces* **2016**, *8*, 4007.
[27] S. H. Kim, J. W. Kim, J. C. Cho, D. A. Weitz, *Lab Chip* **2011**, *11*, 3162.
[28] P. W. Chen, R. M. Erb, A. R. Studart, *Langmuir* **2011**, *28*, 144.
[29] G. Kaufman, R. Boltyanskiy, S. Nejati, A. R. Thiam, M. Loewenberg, E. R. Dufresne, C. O. Osuji, *Lab Chip*, **2014**, *14*, 3494.
[30] N. Madaboosi, K. Uhlig, S. Schmidt, M. S. Jäger, H. Möhwald, C. Duschl, D. V. Volodkin, *Lab Chip*, **2012**, *12*, 1434.
[31] W. J. Duncanson, T. Lin, A. R. Abate, S. Seiffert, R. K. Shah, D. A. Weitz, *Lab Chip*, **2012**, *12*, 2135.
[32] M. B. Chen, S. Srigunapalan, A. R. Wheeler, C. A. Simmons, *Lab Chip*, **2013**, *13*, 2591.
[33] H. Zhang, E. Tumarkin, R. Peerani, Z. Nie, R. M A. Sullan, G. C. Walker, E. Kumacheva, *J. Am. Chem. Soc.* **2006**, *128*, 12205.
[34] F. Bücker, C. Barbosa, P. Quadros, M. Bueno, P. Fiori, C. Huang, A. Frazzon, M. Ferrao, F. Camargo, F. Bento, *Int. Biodeterior. Biodegrad.* **2014**, *95*, 346.
[35] S. Okushima, T. Nisisako, T. Torii, T. Higuchi, *Langmuir* **2004**, *20*, 9905.
[36] T. Nisisako, T. Torii, T. Higuchi, *Lab Chip* **2002**, *2*, 24.
[37] Y. Xia, G. M. Whitesides *Annu. Rev. Mater. Sci.* **1998**, *28*, 153.
[38] A. Utada, E. Lorenceau, D. Link, P. Kaplan, H. Stone, D. Weitz, *Science*, **2005**, *308*, 537.