Liquid-liquid extraction of enzymatically synthesized functional RNA oligonucleotides using reverse micelles with a DNA-surfactant

Maruyama, Tatsuo
Ishizu, Naoki
Eguchi, Yuka
Hosogi, Takuya
Goto, Masahiro

(Citation)
Chemical Communications,52(83):12376-12379

(Issue Date)
2016-10-25

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90004042

Journal Name

COMMUNICATION

Liquid–liquid extraction of enzymatically synthesized functional RNA oligonucleotides using reverse micelles with DNA-surfactant

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

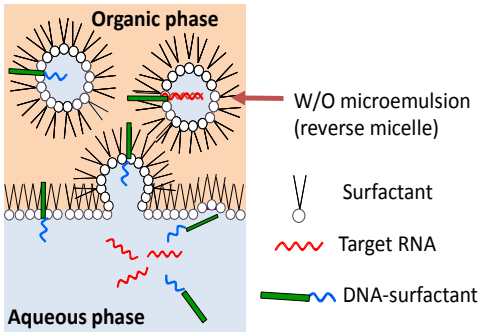
www.rsc.org/

Tatsuo Maruyama,*[a] Naoki Ishizu,[a] Yuka Eguchi,[a] Takuya Hosogi[b] and Masahiro Goto[b]

We successfully implemented solvent extraction of short, single-stranded RNA using reverse micelles (water-in-oil microemulsions) with a DNA-surfactant. A thrombin-binding RNA aptamer was enzymatically synthesized and purified by extraction using the reverse micellar system. The extracted RNA aptamer retained thrombin-binding activity after the extraction procedure.

Since the 1990s, nucleic acid aptamers have received increasing attention.[1-4] Aptamers are short single-stranded RNA or DNA oligonucleotides (15–100-mers) that can bind target molecules with high affinity and specificity. Aptamers have been selected *in vitro* for specific binding to target molecules, which range from small organic compounds to specific cell surfaces.[5] Such aptamers can be chemically or enzymatically synthesized, not requiring use of animals or cell culture systems. In addition, they can be readily integrated with other types of molecular tools, for example, through conjugation with other functional molecules and nanoparticles,[6-9] suggesting that aptamers are potential alternatives to antibodies.[10] Their possible applications include in biosensing, molecular imaging and clinical diagnosis and as drugs or drug carriers.[9, 11-18] There are a number of studies describing discovery of novel aptamers as well as their functions and applications in various fields.[9, 12, 19-22] In fact, in 2004, the US Food and Drug Administration approved an RNA aptamer as a drug for treating neovascular age-related macular degeneration.[23]

Despite the great progress in development of nucleic acid aptamers, available scalable processing techniques for industrial separation and purification of oligonucleotides are unsatisfactory. Several groups reported laboratory-scale techniques for purification of short-stranded DNA and RNA[24-32]



Scheme 1. Schematic illustration of RNA oligonucleotide extraction using DNA-functionalized reverse micelles.

and some purification techniques have been commercialized for laboratory use. However, for industrial applications, there is a strong demand for a scalable purification technique appropriate for mass production of aptamers.

In our previous reports, we investigated solvent extraction of DNA oligonucleotides using reverse micelles (water-in-oil microemulsions) functionalized with a DNA-surfactant, envisioning sequence-specific separation of DNA oligonucleotides.[33,34] A DNA-surfactant is a short single-stranded DNA conjugated to an oleoyl group,[33, 35] enabling transfer of a complementary target DNA to an organic phase in a sequence-selective manner. The water pool of a reverse micelle can dissolve water-soluble compounds and is available as a vehicle for water-soluble compounds in an organic solvent.[36-38] Reverse micellar extraction has the following advantages: i) It can extract water-soluble macromolecules and metal nanoparticles with specific affinities[39] ii) it is scalable; iii) it can suppress microbial contamination in an organic solvent to prevent biological decomposition of oligonucleotides; and iv) it can reduce contamination by undesired biological macromolecules because the reverse micelle serves as an isolated, restricted nanospace.

[a] *Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe 657-8501, Japan. E-mail: tmarutcm@crystal.kobe-u.ac.jp*
[b] *Department of Applied Chemistry, Graduate School of Engineering and Center for Future Chemistry, Kyushu University, 744 Moto-oka, Fukuoka 819-0395, Japan.*
† Electronic supplementary information (ESI) available: The materials, and experimental procedures. See DOI: 10.1039/x0xx00000x

Table 1. Nucleobase sequences of DNA-surfactants and target RNA oligonucleotides.

Name	Sequence
DNA-surfactant **1**	Oleoyl-5′-TCTCCAGCTA-3′
DNA-surfactant **2**	Oleoyl-5′-CCAATACCACATCATCCATA-3
DNA-surfactant **3**	Oleoyl-5′-TTTGGTTAGTTCTCCAGCTATCTTCCAAA-3′
DNA-surfactant **4**	Oleoyl-5′-CCCCCAATACCACATCATCCATATAACTGAAAGCCAAACAGTGGG-3′
Target **1**	FAM[a]-5′-UAGCUGGAGA-3′
Target **2**	FAM -5′- UAUAUGGAUGAUGUGGUAUUGG -3′
Target **3**	FAM-5′-UUUGGAAGAUAGCUGGAGAACUAACCAAA-3′
Target **4**	FAM-5′-CCCACUGUUUGGCUUUCAGUUAUAUGGAUGAUGUGGUAUUGGGGG-3′
Non-thrombin RNA aptamer	FAM-5′-CAUACUUGAAAGUGUAAGGUUGGCGUAUG-3′

[a] FAM, carboxyfluorescein

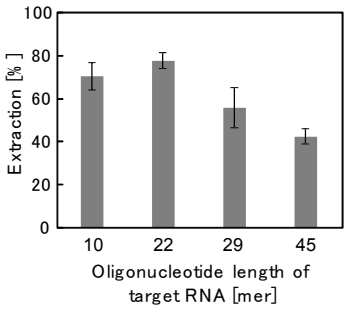


Fig. 1. Effect of sequence length of target RNA on extraction using DNA-surfactants. The aqueous phase contained 25 nM target RNA and the organic phase contained DLPC (10 mM) and 1-hexanol (240 mM). The nucleobase sequences of the target RNAs are listed in Table 1.

Because RNA is very susceptible to enzymatic degradation, its manipulation and processing, in general, requires great care to avoid ribonuclease contamination. We therefore hypothesized that reverse micellar extraction would be appropriate for processing RNA. Our study explored the liquid–liquid extraction of short RNA oligonucleotides using reverse micelles containing a DNA-surfactant, successfully demonstrating forward and backward extraction of RNA oligonucleotides (Scheme 1). We also synthesized an RNA aptamer using T7 RNA polymerase and found that its specific binding properties were retained after the reverse micellar extraction process.

Before investigating RNA extraction, we investigated whether the reverse micellar extraction conditions would prevent contamination with biomolecules. Ribonuclease A (RNase A) was used as a model biomolecule because ribonucleases are ubiquitous and, as contaminants, represent a significant risk in RNA manipulations. Fluorescein-labeled RNase A was prepared. A Tris-HCl buffer solution (10 mM Tris-HCl, pH 8.0, 1 ml) containing 0.4 μg/ml RNase A and 10 mM $MgCl_2$ was mixed with 1 ml isooctane solution containing 10 mM dilauroylphosphatidylcholine (DLPC) and 240 mM 1-hexanol. After this mixture was stirred for 3 h at 25 °C, fluorescence measurements indicated a negligible amount of RNase A (less than 1%) extracted into the reverse micellar phase. This reverse micellar system was composed of phosphatidylcholine and 1-hexanol as surfactants, which are electrostatically neutral and, therefore, did not specifically interact with the RNase A. These results demonstrated that this reverse micellar system could minimize contamination of the organic phase with RNase A.

We anticipated the following mechanism of reverse micellar extraction of RNA: A DNA-surfactant formed an RNA/DNA duplex with its complementary RNA (target RNA) in the aqueous phase (Scheme 1).[33] The RNA/DNA duplex was incorporated into the reverse micelle, resulting in extraction of the target RNA into the organic phase. In our previous studies, a DNA-surfactant complementary, and at molar equivalent amounts, to a target DNA oligonucleotide successfully extracted the target to a reverse micellar phase.[33, 34] Our current study used the same conditions as those in the previous report for reverse micellar extraction of RNA oligonucleotides. We first investigated the reverse micellar extraction of a 10-mer RNA oligonucleotide (target **1,** Table 1) that had been chemically synthesized and purified by high-performance liquid chromatography (HPLC). While no extraction was observed in the absence of DNA-surfactant **1**, the equivalent molar amount of DNA-surfactant **1** (complementary to target **1**) successfully extracted target **1** to an organic phase. The extraction efficiency of target **1** was approximately 70% (Fig. 1), meaning that the DNA-surfactant was similarly effective for extraction of a short RNA oligonucleotide to an organic phase, as it had been for a target DNA molecule.

We next investigated the effect of target RNA length on the extraction (Fig. 1). The DNA-surfactants were designed to be fully complementary to target RNA oligonucleotides of various lengths (Table 1). Among the RNA oligonucleotides tested, the 22-mer RNA oligonucleotide (target **2**) gave the highest extraction efficiency (77%), which was greater than that of the 10-mer RNA oligonucleotide. It was reported that water pools in reverse micelles decreased the melting temperatures of DNA duplex, compared with in a bulk aqueous phase.[38] The short RNA/DNA duplex (10-mer) might be destabilized in the reverse micellar phase to some extent, which would result in an extraction efficiency lower than that of the 22-mer RNA/DNA duplex.

Although an increase in the RNA length above a 22-mer decreased extraction efficiency, over 40% of a 45-mer RNA oligonucleotide (target **4**) was, nonetheless, successfully extracted. Dynamic light scattering measurements showed

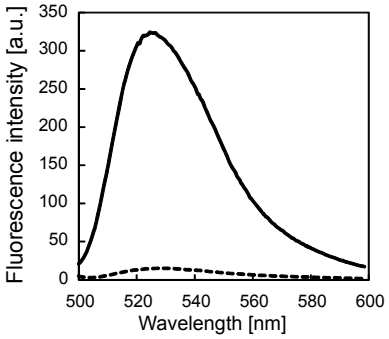


Fig. 2. Fluorescence spectra of enzymatically synthesized RNA with SYBR Green II.

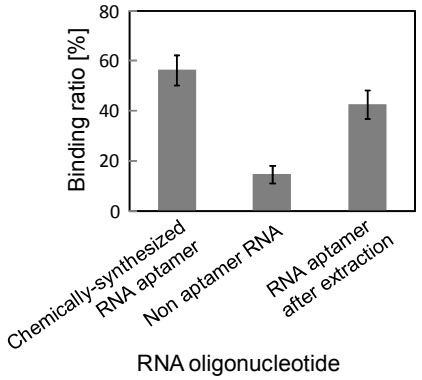


Fig. 3. Binding activities of an RNA aptamer (target **3**) and a non-aptamer RNA to immobilized thrombin. The chemically synthesized RNA aptamer was purified by HPLC. The RNA aptamer after extraction represents the enzymatically synthesized RNA aptamer, purified by the reverse micellar extraction system.

that the diameter of the reverse micelle involving DLPC was 16.7 nm, indicating that, because the length of DLPC is greater than 2 nm, the water pool was smaller than 16.7 nm. It would be difficult, therefore, to encapsulate a long RNA/DNA duplex inside the restricted water pool of the reverse micelle, explaining the low percent extraction of the longer target RNA. Indeed, the physical length of a 45 base-pair DNA duplex was estimated as approximately 16 nm, comparable to the diameter of the reverse micelle.

The extracted target RNA should ideally be back-extracted to an aqueous solution and isolated from the DNA-surfactant, consistent with our overall goal of purifying RNA oligonucleotides. Addition of 2-butanol to the reverse micellar system at 80 °C destroyed the micelles and enabled 90 ± 4 % back-extraction of target **3** (29-mer) from an organic phase to an aqueous phase.

The back-extracted target RNA was still in a duplex with the DNA-surfactant. To isolate the single-stranded target RNA from the DNA-surfactant, a Tris-HCl buffer solution containing back-extracted target **3** and DNA-surfactant **3** (15 nM each) was mixed with a fresh isooctane solution containing 10 mM DLPC and 240 mM 1-hexanol. The mixture, in two phases, was stirred at 80 °C for 3 h to selectively extract DNA-surfactant **3** to the organic phase (reverse micellar phase). The melting temperature of the RNA/DNA duplex (target **3**) was 74 °C in the Tris-HCl buffer solution, so the target **3** was not hybridized with DNA-surfactant **3** under these 80 °C conditions. The reverse micellar system, at 80 °C, extracted more than 85% of DNA-surfactant **3** to the organic phase, with 89 ± 4 % of target **3** remaining in the aqueous phase. The back-extraction and isolation procedures successfully yielded target **3**, consistent with our previous results.[34] Other RNA oligonucleotides (targets **1**, **2** and **4**) were also back-extracted to an aqueous phase, each at approximately 90% yield, under the same conditions.

For experiments described so far, we first used chemically synthesized, HPLC-purified RNA oligonucleotides. We then enzymatically synthesized an RNA oligonucleotide (target **3**) using T7 RNA polymerase and a DNA duplex template (oligo DNA A/oligo DNA B, Table S1).[40] Next, we tested extraction of the enzymatically-synthesized RNA oligonucleotide using the reverse micellar system. Fig. 2 shows fluorescence spectra of RNA synthesis solutions with and without T7 RNA polymerase. In the presence of T7 RNA polymerase, fluorescence was high, indicating successful synthesis of the RNA oligonucleotide. The yield of the RNA oligonucleotide (target **3**) was 1 μg.

The enzymatically-synthesized target **3** was subjected to reverse micellar extraction. The percent extraction was 42 ± 2 %, somewhat lower than that for the chemically-synthesized RNA (Fig. 1). The presence of enzyme, DNA duplex and nucleobases might have affected extraction efficiency. The extracted target **3** was back-extracted and isolated from DNA-surfactant **3** as described for the chemically synthesized oligonucleotides, resulting in a yield of 36 ± 7 % of target **3** in the aqueous solution. These results demonstrated that an enzymatically synthesized RNA oligonucleotide could be forward- and back-extracted using the reverse micellar system with a DNA-surfactant. It should be noted that the back-extracted solution did not contain detectable protein, indicating that potentially contaminating RNA-synthesis related proteins had been removed by the reverse micellar extraction.

There are several reports that biomacromolecules, especially proteins, were denatured or deactivated through reverse micellar extraction because of their strong interactions with the oil/water interface.[41-43] To address this concern, we investigated the functional properties of enzymatically synthesized target **3** after the reverse micellar extraction process. The sequence of target **3** was identical to that of the thrombin-binding RNA-aptamer.[40] Its binding activity was therefore was evaluated using thrombin-immobilized Sepharose beads, as previously described.[34] Chemically-synthesized target **3**, which was purified by HPLC (without reverse micellar extraction), bound to immobilized thrombin (Fig. 3). As a negative control, a non-thrombin RNA oligonucleotide (29-mer, Table 1) showed low thrombin binding under the same conditions. Enzymatically-synthesized target **3**, after reverse micellar extraction and isolation, also bound to immobilized thrombin. The binding ratio of the enzymatically-synthesized target **3** to thrombin was slightly lower than that of chemically-synthesized molecule, potentially caused by small amounts of DNA-surfactant **3** remaining after the isolation. These results showed that an enzymatically synthesized RNA aptamer was not degraded,

maintaining its intrinsic binding activity, during the reverse micellar extraction and isolation processes.

In conclusion, we first achieved reverse micellar extraction of RNA oligonucleotides using a DNA-surfactant. RNA oligonucleotides with nucleobase lengths from 10- to 45-mers were forward-extracted to an organic phase. The extracted RNA oligonucleotides were then back-extracted to an aqueous phase. We enzymatically synthesized a 29-mer RNA aptamer using T7 RNA polymerase. The enzymatically synthesized RNA aptamer was also extracted by the reverse micellar system and then readily back-extracted to an aqueous phase. The RNA aptamer, which had undergone forward- and back-extraction followed by isolation from the DNA-surfactant, had thrombin binding activity comparable to that of intrinsic RNA aptamer. Our study demonstrated that reverse micellar extraction using a DNA-surfactant can be used to purify a functionally intact RNA oligonucleotide.

This work was supported partially by Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), MEXT, Japan, and by JSPS KAKENHI Grant Number 16H04577.

Notes and references

1 A. D. Keefe, S. Pai and A. Ellington, Nat. Rev. Drug Discov., 2010, 9, 537-550.
2 A. B. Iliuk, L. H. Hu and W. A. Tao, Anal. Chem., 2011, 83, 4440-4452.
3 G. Aquino-Jarquin and J. D. Toscano-Garibay, Int. J. Mol. Sci., 2011, 12, 9155-9171.
4 F. Radom, P. M. Jurek, M. P. Mazurek, J. Otlewski and F. Jelen, Biotechnol. Adv., 2013, 31, 1260-1274.
5 M. Blank and M. Blind, Curr. Opin. Chem. Biol., 2005, 9, 336-342.
6 C. M. Niemeyer, Chem. Eur. J., 2001, 7, 3188-3195.
7 A. Saghatelian, K. M. Guckian, D. A. Thayer and M. R. Ghadiri, J. Am. Chem. Soc., 2003, 125, 344-345.
8 C. M. Niemeyer, Biochem Soc Trans, 2004, 32, 51-53.
9 J. H. Lee, M. V. Yigit, D. Mazumdar and Y. Lu, Adv. Drug Deliv. Rev., 2010, 62, 592-605.
10 S. D. Jayasena, Clin Chem, 1999, 45, 1628-1650.
11 V. Bagalkot, L. Zhang, E. Levy-Nissenbaum, S. Jon, P. W. Kantoff, R. Langer and O. C. Farokhzad, Nano. Lett., 2007, 7, 3065-3070.
12 M. Famulok, J. S. Hartig and G. Mayer, Chem. Rev., 2007, 107, 3715-3743.
13 J. Shimada, T. Maruyama, T. Hosogi, J. Tominaga, N. Kamiya and M. Goto, Biotechnol Lett, 2008, 30, 2001-2006.
14 D. Kim, Y. Y. Jeong and S. Jon, ACS Nano., 2010, 4, 3689-3696.
15 J. Shimada, T. Maruyama, M. Kitaoka, H. Yoshinaga, K. Nakano, N. Kamiya and M. Goto, Chem. Commun., 2012, 48, 6226-6228.
16 G. Z. Zhu, L. Meng, M. Ye, L. Yang, K. Sefah, M. B. O'Donoghue, Y. Chen, X. L. Xiong, J. Huang, E. Q. Song and W. H. Tan, Chem. Asian J., 2012, 7, 1630-1636.
17 M. Ashrafuzzaman, Biomed. Res. Int., 2014.
18 G. Zhu, G. Niu and X. Chen, Bioconjug. Chem., 2015, 26, 2186-2197.
19 A. D. Ellington and J. W. Szostak, Nature, 1990, 346, 818-822.
20 C. Tuerk and L. Gold, Science, 1990, 249, 505-510.
21 A. D. Ellington and J. W. Szostak, Nature, 1992, 355, 850-852.
22 S. Tombelli, A. Minunni and A. Mascini, Biosens. Bioelectron., 2005, 20, 2424-2434.
23 E. W. Ng, D. T. Shima, P. Calias, E. T. Cunningham, Jr., D. R. Guyer and A. P. Adamis, Nat Rev Drug Discov, 2006, 5, 123-132.
24 J. A. Bantle, I. H. Maxwell and W. E. Hahn, Anal. Biochem., 1976, 72, 413-427.
25 D. Werner, Y. Chemla and M. Herzberg, Anal. Biochem., 1984, 141, 329-336.
26 E. Hornes and L. Korsnes, Genet Anal-Biomol E, 1990, 7, 145-150.
27 T. Ito, C. L. Smith and C. R. Cantor, Proc. Natl. Acad. Sci. USA, 1992, 89, 495-498.
28 K. Kuribayashiohta, S. Tamatsukuri, M. Hikata, C. Miyamoto and Y. Furuichi, Biochim. Biophys. Acta, 1993, 1156, 204-212.
29 D. Umeno, T. Mori and M. Maeda, Chem. Commun., 1998, 1433-1434.
30 P. Kohli, C. C. Harrell, Z. H. Cao, R. Gasparac, W. H. Tan and C. R. Martin, Science, 2004, 305, 984-986.
31 L. E. Easton, Y. Shibata and P. J. Lukavsky, RNA, 2010, 16, 647-653.
32 M. Zlobina, O. Sedo, M. Y. Chou, L. Slepankova and P. J. Lukavsky, Biotechniques, 2016, 60, 75-83.
33 T. Maruyama, T. Hosogi and M. Goto, Chem. Commun., 2007, 4450-4452.
34 T. Maruyama and N. Ishizu, ChemNanoMat, 2016, 2, 461-465.
35 T. Maruyama, H. Yamamura, M. Hiraki, Y. Kemori, H. Takata and M. Goto, Colloids Surf. B, 2008, 66, 119-124.
36 K. E. Goklen and T. A. Hatton, Biotechnol. Progr., 1985, 1, 69-74.
37 M. Goto, A. Momota and T. Ono, J. Chem. Eng. Jpn., 2004, 37, 662-668.
38 T. Maruyama, L. C. Park, T. Shinohara and M. Goto, Biomacromolecules, 2004, 5, 49-53.
39 T. Kato, Y. Fujimoto, A. Shimomura and T. Maruyama, RSC Adv, 2014, 4, 57899-57902.
40 X. Liu, G. Cao, H. Ding, D. Zhang, G. Yang, N. Liu, M. Fan, B. Shen and N. Shao, FEBS Lett., 2004, 562, 125-128.
41 M. E. Leser and P. L. Luisi, Chimia, 1990, 44, 270-282.
42 R. Kuboi, K. Hashimoto and I. Komasawa, Kagaku Kogaku Ronbunshu, 1990, 16, 335-342.
43 L.-J. Chen and X.-J. Cao, J. Chem. Eng. Jpn., 2007, 40, 511-515.

Electric supplementary information

Liquid–liquid extraction of enzymatically synthesized functional RNA oligonucleotides using reverse micelles with DNA-surfactant

Tatsuo Maruyama,*[a] Naoki Ishizu,[a] Yuka Eguchi,[a] Takuya Hosogi[b] and Masahiro Goto[b]

[a]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe 657-8501, Japan

[b]Department of Applied Chemistry, Graduate School of Engineering and Center for Future Chemistry, Kyushu University, 744 Moto-oka, Fukuoka 819-0395, Japan

Experimental Section

Materials

Synthesized DNA oligonucleotides were from Tsukuba Oligo Service Co., Ltd (Ibaraki, Japan). Synthesized RNA oligonucleotides were from Japan Bio Services Co., Ltd. (Saitama, Japan). The nucleobase sequences are listed in Table 1. Tris-HCl buffer (10 mM, pH 8) containing 1 mM EDTA was from Fluka. Thrombin from bovine plasma was from Sigma-Aldrich (St Louis, MO). DLPC and other chemicals were from Wako Pure Chemicals (Osaka, Japan).

Reverse micellar extraction of ribonuclease A

Sodium bicarbonate buffer (pH 9.0, 0.1 M, 1 ml) containing 1 mg/ml RNase A was mixed with dimethyl sulfoxide solution (0.1 ml) containing 10 mg/ml fluorescein isothiocyanate and stirred at 4 °C

for 8 h. Fluorescein-labeled RNase A was purified twice over a PD-10 column. Fluorescein-labeled RNase A was eluted with Tris-HCl buffer (10 mM, pH 8) containing 1 mM EDTA.

Reverse micellar extraction was performed as follows. The aqueous phase was Tris-HCl buffer (pH 8, 10 mM) containing 1 mM EDTA, 10 mM $MgCl_2$ and fluorescein-labeled RNase A (0.4 μg/ml). The organic phase was isooctane containing DLPC (10 mM) and 1-hexanol (240 mM). The organic phase (1 mL) was mixed with the aqueous phase (1 mL) in a glass vial. The two phases were gently stirred with a magnetic stir bar at 25 °C for 3 h. The fluorescence of each phase was measured with a luminescence spectrometer, model LS 50B (PerkinElmer, Waltham, MA) at 25 °C to determine the concentration of fluorescein-labeled RNase A in each phase. Excitation and emission wavelengths were 494 and 520 nm, respectively.

Synthesis of DNA-surfactants

The DNA-surfactant was synthesized as previously reported.[1,2] Briefly, 5′-aminated DNA oligonucleotide (0.5 mM) in phosphate buffer (50 mM, 10 μl) was mixed with a dimethylsulfoxide solution (50 μl) containing oleic acid *N*-hydroxysuccinimide ester (1 mM) at 40 °C for 24 h. The synthesized 5′-oleoyl DNA oligonucleotides (DNA-surfactants) were purified using a high performance liquid chromatograph (Shimadzu LC20 system, Kyoto, Japan) equipped with a C18 column (4 × 250 mm, GL Sciences, Tokyo), initially eluted with 10% acetonitrile in 0.1 M triethylamine acetate buffer for 5 min, then with a linear gradient of 10 to 90% acetonitrile over 30

min and isocratic 90% acetonitrile for 10 min at a flow rate of 1.0 ml/min.

Extraction of target RNA using reverse micelles and a DNA-surfactant

The aqueous phase was a Tris-HCl buffer (pH 8, 10 mM) containing 1 mM EDTA, 10 mM $MgCl_2$, a fluorophore-labeled target RNA (25 nM) and the DNA-surfactant (25 nM). The organic phase was isooctane containing DLPC (10 mM) and 1-hexanol (240 mM). The organic phase (1 mL) was mixed with the aqueous phase (1 mL) in a glass vial. The two phases were gently stirred using a magnetic stir bar at 25 °C for 3 h, taking care not to disrupt the interface between the two phases. The fluorescence of each phase was measured in a luminescence spectrometer, model LS 50B (PerkinElmer) at 25 °C to determine the concentration of the target RNA in each phase. Excitation and emission wavelengths used to detect the carboxyfluorescein (FAM) fluorophore were 494 and 520 nm, respectively. All experiments were performed in triplicate and error bars in the figures represent standard deviations.

The diameter of reverse micelles was measured using a Zetasizer Nano ZS (Malvern, Worcestershire, UK).

Back-extraction of extracted RNA to an aqueous phase

A DNA-surfactant conjugated with FITC at its 3′ end and an unlabeled target RNA were used for back-extraction to monitor the concentration of the DNA-surfactant. To monitor the back-extraction of target RNA, a FAM-labeled target RNA and a DNA-surfactant without FITC were used. After the

reverse micellar extraction, as described above, the organic phase (1 mL) containing the target RNA was mixed with Tris-HCl buffer (1 mL) containing 1 mM EDTA, followed by addition of 0.5 mL 1-butanol at 75 °C to destroy the reverse micelles. The mixture was stirred and then centrifuged to collect the aqueous phase. The aqueous phase contained duplexes of the target RNA and the DNA-surfactant. To remove the DNA-surfactant, the aqueous phase was mixed with isooctane containing DLPC (10 mM) and 1-hexanol (240 mM) at 80 °C. The reverse micelles formed in the organic phase extracted the DNA-surfactant, resulting in single-stranded target RNA separated from DNA-surfactant. At each step, fluorescence of the two phases was measured to determine concentrations of each component.

Enzymatic synthesis of an RNA aptamer

Oligo DNAs A and B (Table S1) were dissolved in Tris-HCl buffer (pH 8, 10 mM) containing 1 mM EDTA and 50 mM potassium acetate to prepare a DNA template solution containing each Oligo DNA at 10 μM. Using the DNA template solution (4 μl) and T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Fitchburg, WI), target 3 was enzymatically synthesized according to the manufacturer's instructions. The synthesized target 3 was used for the following experiments without removal of the DNA template or enzymes. The concentration of synthesized target 3 was determined using SYBR Green II. Excitation and emission wavelengths were 497 and 520 nm, respectively. The amount of synthesized target **3** was determined using a Qubit RNA assay kit (Thermo Fisher Scientific, Waltham, MA).

Assay of thrombin and RNA-aptamer binding

N-Hydroxysuccinimidyl (NHS)-activated Sepharose (700 μL, GE Healthcare, Little Chalfont, UK) was centrifuged and the supernatant removed. A phosphate buffer solution (pH 8, 100 mM, 700 μL) containing 0.2 mg/mL thrombin was mixed with the NHS-activated Sepharose at 25 °C for 3 h. The reaction mixture was centrifuged and the supernatant removed. The collected thrombin-immobilized beads were then mixed with a Tris-HCl buffer solution (700 μL) containing 15 nM thrombin-binding RNA aptamer (synthesized enzymatically and purified by reverse micellar extraction), 100 mM NaCl and 100 mM KCl for 3 h. After centrifugation, the supernatant was labeled with SYBR Green II and its fluorescence measured to determine the concentration of the unbound RNA aptamer. The binding ratio of RNA aptamer to thrombin was calculated from the concentration of the unbound RNA aptamer. Because the non-thrombin aptamer was labeled with carboxyfluorescein (FAM), the concentration of the unbound non-thrombin aptamer was determined by measuring FAM fluorescence. Excitation and emission wavelengths used to detect the FAM fluorophore were 494 and 520 nm, respectively.

References

1. J. Shimada, T. Maruyama, M. Kitaoka, H. Yoshinaga, K. Nakano, N. Kamiya and M. Goto, *Chem. Commun.*, 2012, **48**, 6226-6228.

2. T. Maruyama, H. Yamamura, M. Hiraki, Y. Kemori, H. Takata and M. Goto, *Colloids Surf B Biointerfaces,* 2008, **66**, 119-124.

Table S1. Sequences of template DNAs for enzymatic RNA synthesis

Name	Sequence
Oligo DNA A	5′-TAATACGACTCACTATATTTGGAAGATAGCTGGAGAACTAACCAAA-3
Oligo DNA B	3′-ATTATGCTGAGTGATATAAACCTTCTATCGACCTCTTGATTGGTTT-5′