Bioorganic & Medicinal Chemistry Letters 23 (2013) 2675–2678

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

An immunoassay utilizing the DNA-coated polydiacetylene micelles as a signal generator

Hoa Thi Hoang [a,b], Taemin Lee [c], Byeong-Su Kim [c], Ki-Cheol Han [a], Dae-Ro Ahn [a,b,*]

[a] Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Hwarangno 14-gil, Seongbuk-gu, Seoul 136 791, Republic of Korea
[b] KIST Campus, University of Science and Technology, Hwarangno 14-gil, Seongbuk-gu, Seoul 136 791, Republic of Korea
[c] Interdisciplinary School of Green Energy and KIER-UNIST Advanced Center for Energy, Ulsan National Institute of Science and Engineering (UNIST), Ulsan 689 798, Republic of Korea

ARTICLE INFO

Article history:
Received 6 December 2012
Revised 15 February 2013
Accepted 20 February 2013
Available online 1 March 2013

Keywords:
Polydiacetylene (PDA)
Micelles
Immunoassay
Fluorescence intensity
Alpha-fetoprotein (AFP)

ABSTRACT

Immunoassay is an important technique to detect the disease biomarkers and pathogenic biological agents which often present at low levels in clinical samples. To improve sensitivity of the immunoassay, here we described the DNA-coated, nano-sized micelles in which the DNA strands play a role as signal generators in an immunoassay. This micelle-based immunoassay was evaluated for quantitation of a liver cancer biomarker and the sensitivity of the method was compared with those of the conventional methods.

Enzyme-linked immunosorbent assay (ELISA) is the conventional immunoassay technique based on a specific interaction between antigens and antibodies.[1,2] This method has been regarded as a gold standard in detection of disease-relating biomarkers in clinical area and used for the detection and quantitation of various biomolecules such as peptides, proteins, antibodies, and hormones.[3–6] Despite these wide applications, ELISA is often limited because of its relatively low detection sensitivity. Recently, we have developed a microwell plate-based immunoassay, called oligonucleotide-linked immunosorbent assay (OLISA).[7] OLISA utilizes DNA oligonucleotides conjugated on detection antibodies (dAbs) that mediated the cleavage of fluorogenic RNA probes by RNase H for generation of the fluorescent detection signal (Fig. 1B, left). Since the DNA can play a dual role as the signal generator and the barcode for a detection antibody, OLISA can be used in a multiplexed assay, while ELISA is unable to detect multiple targets in a microwell. On the consideration that the DNA strand is the template for the signal generation, the increased number of DNA strands per dAb in the DNA–dAb conjugate would improve the detection sensitivity. In that regard, nanomaterials having multiple reactive sites in a restricted surface area are potentially useful cross-linkers for connecting multiple DNA strands to a dAb, leading to improved detection sensitivity in immunoassays.[8]

Self-assembled constructs made of diacetylene surfactants have gained popularity since they undergo polymerization via a

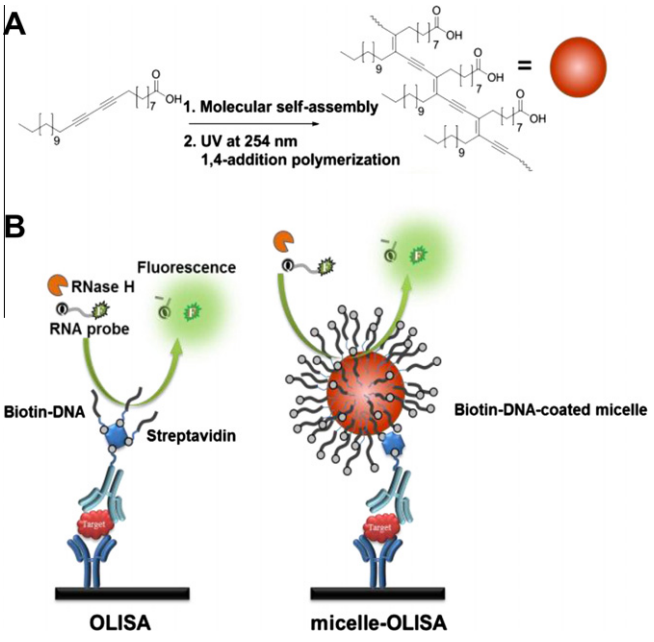

Figure 1. (A) The chemical structure of photo-crosslinked PDA micelles. (B) Schematic presentation of OLISA (left) and micelle-OLISA (right).

* Corresponding author. Tel.: +82 2 958 6645; fax: +82 2 958 5909.
E-mail address: drahn@kist.re.kr (D.-R. Ahn).

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.02.087

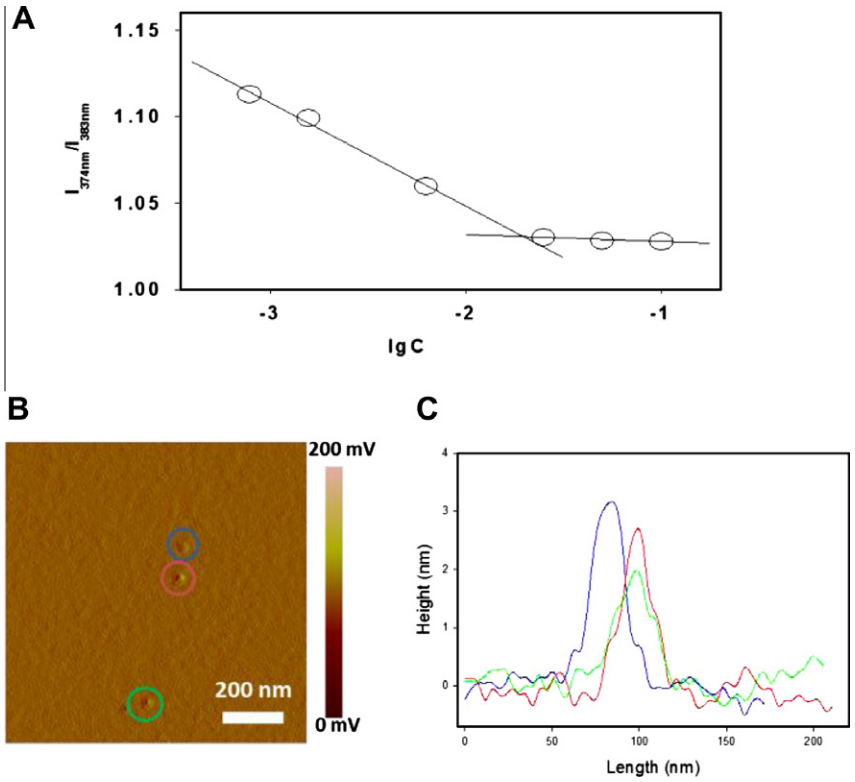

Figure 2. (A) CMC of PDA micelles. (B) The AFM image of PDA micelles. (C) Heights of micelles indicated by circles in Figure 2B.

topochemical 1,4-addition mechanism by UV radiation at 254 nm to become relatively stable structures (Fig. 1A). This photopolymerized polydiacetylene (PDA) provides various attractive supramolecular structures including vesicles and micelles.[9–11] While conjugated PDA vesicles have been intensively investigated as sensors to detect chemically and biologically interesting molecules,[12–16] the PDA-based micelles have appeared in limited applications.[17–20] As an effort to utilize PDA-based micelles in development of an immnoassay, multiple DNA strands were loaded on a PDA micelle by utilizing multiple carboxylic acid groups on the surface of the micelle in this study (Fig. 1B, right). This DNA-coated PDA micelle was further conjugated with a dAb and used in the OLISA assay for detection of a liver cancer marker, α-feto-protein (AFP). The detection sensitivity of our micelle-OLISA was examined carefully in comparison with OLISA and ELISA. Further, the accuracy and reproducibility of the assay were also evaluated by detecting the target antigen in human sera.

To prepare the PDA micelle, the critical micelle concentration (CMC) of the monomer (10,12-pentacosadiynoic acid) was determined by following the literature procedure using pyrene as a fluorescence probe.[19] After dispersion of the monomer in the presence of pyrene, the ratiometric values of the fluorescence intensity of pyrene at two different wavelengths (373 and 383 nm) were plotted versus the concentration of the monomer (Fig. 2A). The CMC value of the micelles determined from the intersection of two curves was 20 mg/L or 53 μM. The PDA micelles were thus prepared by dispersion of the monomer at a higher concentration than the CMC value in NaOH solution (pH 12) and subsequent sonication at 60 °C.[21] The structure of micelles was further stabilized by the photo-crosslinking performed with UV irradiation at 254 nm for 5 h. As the result, yellowish solution of the photo-polymerized micelles was obtained, which was consistent with the previous reports on the photoconjugated PDA micelles (Fig. S1, Supplementary data).[20,22–24] According to the AFM measurement,

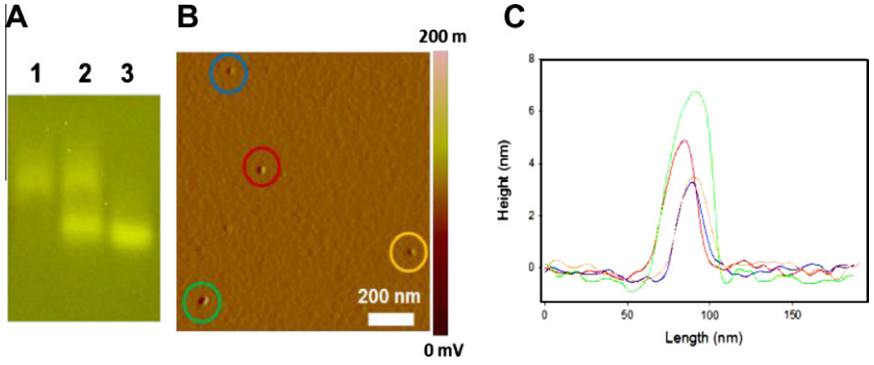

Figure 3. Characterization of DNA-coated micelles. (A) Electrophoresis of reaction mixtures with different blend ratios of DNA and the micelle (lane 1: 20–1, lane 2: 50–1, lane 3: DNA alone) was performed to estimate the conjugation yield on 3% agarose gel pre-stained with SYBR gold. (B) The AFM image of DNA-coated micelles. (C) Heights of micelles indicated by circles in Figure 3B.

the micelles showed a spherical morphology with heights of 2–3 nm (Fig. 2B and C).

The polymerized micelles were chemically coupled with DNA strands containing an amine moiety at one end and a biotin unit at the other end. The amide coupling reaction was performed in 0.1 M MES buffer at pH 5 by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The conjugation reaction was analyzed by agarose gel electrophoresis. As shown in Fig. 3A, the DNA-coated micelles (lane 1 and 2) were moving more slowly than the unreacted DNA strand (lane 3), suggesting that the DNA-coated micelle was successfully prepared. Although the aggregation number of the micelle was assumed as 100 monomers per one particle according to the literature,[25] when the DNA was used in excess (50 times) compared to the concentration of the micelle, the unreacted DNA strands still remained in the reaction solution (lane 2). These unreacted DNA strands might lower the sensitivity of the assay, because these unreacted DNA strands themselves could also be conjugated with dAbs through the specific streptavidin–biotin interaction during immunoassays. After attempting several different ratios between the DNA and the micelle, we found the suitable molar ratio between the DNA and the micelle (20:1) at which all the DNA strands used were coupled with the micelle in the reaction (lane 1). The size of the DNA-coated micelle was estimated as 3–6 nm in height by AFM, which is slightly larger than that of the plain micelle (Fig. 3B and C).

After preparation of the DNA-coated micelle, OLISA was conducted to quantitatively detect a liver cancer marker, α-fetoprotein (AFP), in buffer solutions. To evaluate the assay performance of micelle-OLISA, the capture antibody (cAb) for AFP was coated on wells of black 96-microwell plate, and then serially diluted biomarker solutions were added into each well after blocking with phosphate buffered saline (PBS) containing 3% BSA. The biotinylated dAb and streptavidin were sequentially added to each well. The DNA-coated micelle solution was prepared by diluting the assay buffer (PBS containing 1% BSA and 0.1% Tween 20) to a final concentration of 15 nM and incubated for 1 h at room temperature after being added into each well to conjugate dAb with the DNA-coated micelle via biotin–streptavidin interaction. Then, the RNase H reaction was carried out with the fluorogenic RNA probe for 1 h at 37 °C, and fluorescence intensities were measured by a fluorescence microplate reader. As shown in Fig. 4, the fluorescence intensity was linearly proportional to the AFP concentration in the range from 0 to 10 ng/mL. The limit of detection (LOD) value obtained from the curve was 0.16 ng/mL.

To compare these results obtained by micelle-OLISA with those by previous methods, OLISA and ELISA were also performed to construct the quantitation curves, and LOD values were determined as shown in Table 1. The micelle-OLISA showed approximately four to fivefold higher sensitivity than OLISA and ELISA. The improved sensitivity was obviously obtained by employing the PDA-micelle as the carrier of multiple DNA strands of which each strands could work as a signal generator. The level of improvement in the sensitivity was, however, not as high as expected from the increased number of DNA strands per dAb (20 strands) in micelle-OLISA compared to that in OLISA (up to 3 strands). We believe that some of the DNA strands immobilized on the micelle surface might not be active for the signal generation reaction because of hindrance by other neighboring DNA strands and the solid phase of the microwell.

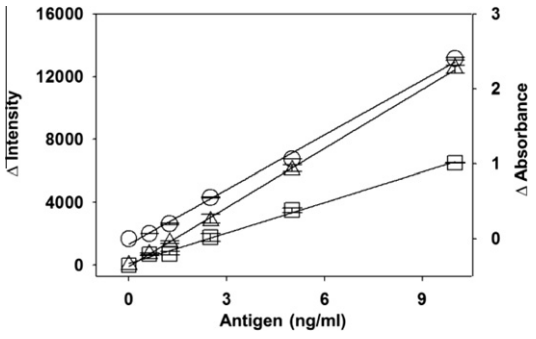

Figure 4. Immunoassays of AFP using ELISA (○), OLISA (□), and micelle-OLISA (△). The detection signals were absorbance for ELISA or fluorescence for OLISA and micelle-OLISA, respectively.

Table 1
Limit of detection (LOD) of ELISA, OLISA, and micelle-OLISA in AFP detection

Method	LOD (ng/mL)
ELISA	0.77
OLISA	0.69
Micelle-OLISA	0.16

Table 2
Precision (%CV) and accuracy (%recovery) of micelle-OLISA and ELISA

	AFP (ng/mL)	20	40	80
Micelle-OLISA	%CV	6.7	3.1	3.9
	%Recovery	89.8	90.1	90.2
ELISA	%CV	1.2	2.0	5.2
	%Recovery	97.7	99.4	95.3

To further test the potential of the micelle-OLISA in clinical applications, the precision (%CV) and the accuracy (%recovery) values of the method were determined for the biomarker in human sera. Eighteen healthy human serum samples containing AFP (20, 40 or 80 ng/mL) were analyzed by using micelle-OLISA. As presented in Table 2, the %CV values were less than 10% at all concentrations examined, demonstrating that micelle-OLISA can be reliable method for clinical uses. The %recovery expressing the accuracy of the assay ranged from 89.8% to 90.2%, indicating that the method could be used potentially for practical samples.[26]

In summary, we have prepared the DNA-coated PDA micelle and employed the micelle in an immunoassay system, called micelle-OLISA utilizing the PDA-micelle for conjugating dAb with multiple signal generating DNA strands. Compared with the previous methods such as ELISA and OLISA, the micelle-OLISA showed improved sensitivity in quantitation of AFP in buffer solution. In addition, the micelle-OLISA showed acceptable levels of accuracy and reproducibility that are required for practical applications. We therefore expect that the micelle-OLISA presented in this study can be useful for clinical diagnosis as well as research applications.

Acknowledgments

This study was supported by a grant funded by Korea Institute of Science and Technology, a grant of the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (2011-0009172), grants of the Korea Health technology R&D project, Ministry of Health & Welfare, Republic of Korea (A090147 and A121191), and a grant the Proteogenomic Research Program through the National Research Foundation of Korea funded by the Korean Ministry of Education, Science and Technology.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.087.

References and notes

1. Engvall, E.; Perlmann, P. *Immunochemistry* **1971**, *8*, 871.
2. Kohler, C.; Milstein, C. *Nature* **1975**, *256*, 495.
3. Hnasko, R.; Lin, A.; McGarvey, J. A.; Stanker, L. H. *Clin. Virol.* **2001**, *22*, 41.
4. Hnasko, Robert; Lin, Alice; Jeffery, A.; McGarvey; Larry, H.; Stanker *Biochem. Biophys. Res. Commun.* **2011**, *410*, 726.
5. Suzaki, Yuki; Ozawa, Yuri; Kobori, Hiroyuki *Peptides* **2006**, *27*, 3000.
6. Swart, N.; Pool, E. *J. Immunoassay Immunochem.* **2007**, *28*(4), 395.
7. Han, K.; Ahn, D.; Yang, E. *Bioconjugate Chem.* **2010**, *21*, 2190.
8. Han, K.; Yang, E.; Ahn, D. *Chem. Commun.* **2012**, *48*, 5895.
9. Kim, J. M.; Lee, J. S.; Choi, H.; Sohn, D.; Ahn, D. J. *Macromolecules* **2005**, *38*, 3966.
10. Ma, Z.; Ren, J. *Colloids Surf.* **2007**, *303*, 179.
11. Howarth, N. M.; Lindsell, W. E.; Murray, E.; Preston, P. N. *Tetrahedron Lett.* **2003**, *44*, 8089.
12. Reppy, M. A.; Pindzola, B. A. *Chem. Commun.* **2007**, 4317.
13. Lee, J.; Kim, H. *J. Am. Chem. Soc.* **2008**, *130*, 5010.
14. Schrader, T.; Zadmard, R. *J. Am. Chem. Soc.* **2004**, *126*, 7752.
15. Kolusheva, S.; Zadmard, R.; Schrader, T.; Jelinek, R. *J. Am. Chem. Soc.* **2006**, *128*, 13592.
16. Reichert, A.; Nagey, J. O.; Spevak, W.; Charych, D. *J. Am. Chem. Soc.* **1995**, *117*, 829.
17. Choi, H.; Bae, Y. P.; Yu, G. S.; Huh, K. M.; Choi, J. S. *J. Nanosci. Nanotechnol.* **2008**, *8*, 5104.
18. Kim, Y. H.; Subramanyam, E.; Kim, J. H.; Huh, K. M.; Choi, H.; Choi, J. S.; Choi, Y. H.; Lee, Y. K.; Park, S. W. *J. Nanosci. Nanotechnol.* **2010**, *10*, 3275.
19. Wilhelm, M.; Zhao, C. L.; Wang, Y.; Xu, R.; Winnik, M. A.; Mura, J. L.; Riess, G.; Croucher, M. D. *Macromolecules* **1991**, *24*, 1033.
20. Mackiewicz, Nicolas; Surendran, Geetarani; Remita, Hynd; Keita, Bineta; Zhang, Guangjin; Nadjo, Louis; Hagège, Agnès; Doris, Eric; Mioskowski, Charles *J. Am. Chem. Soc.* **2008**, *130*, 8110.
21. Ogier, Julien; Arnauld, Thomas; Carrot, Géraldine; Lhumeau, Antoine; Delbos, Jean-Marie; Boursier, Claire; Loreau, Olivier; Lefoulon, Francois; Dori, Eric *Org. Biomol. Chem.* **2010**, *8*, 3902.
22. Sixl, H.; Kollmar, C.; Huber, R.; Sigmund, E. *Phys. Rev. B* **1987**, *36*, 2747.
23. Kollmar, C. *J. Chem. Phys.* **1993**, *98*, 7210.
24. Itoh, C.; Kondoh, T.; Tanimura, K. *J. Phys. Soc. Jpn.* **1999**, *68*, 1711.
25. Gravel, E.; Doris, E. *Chem. Eur. J.* **2012**, *18*, 400.
26. Findlay, J. W.; Smith, W. C.; Lee, J. W.; Nordblom, G. D.; Das, I.; SeSilva, B. S.; Khan, M. N.; SeSilva, R. R. *J. Pharm. Biomed. Anal.* **2000**, *21*, 1249.