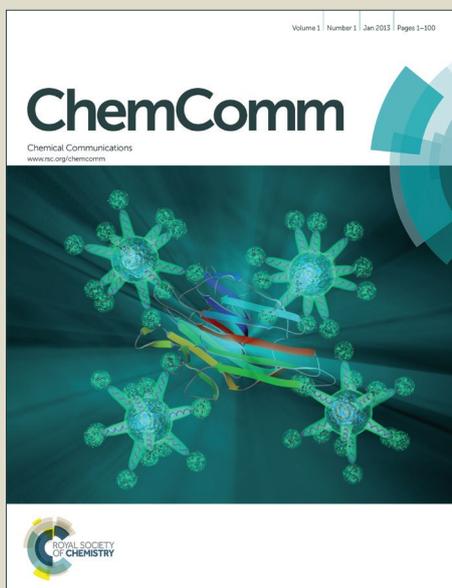


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COMMUNICATION

A Simple Modular Aptasensor Platform Utilizing Cucurbit[7]uril and Ferrocene Derivative As an Ultrastable Supramolecular Linker

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www.rsc.org/Don-Wook Lee^{†a,b}, Kyeng Min Park^{†a}, Bokyoung Gong^{a,c}, Dinesh Shetty^a, Jayshree K. Khedkar^a, Kangkyun Baek^a, Jeehong Kim^{a,c}, Sung Ho Ryu^{b,e*}, and Kimoon Kim^{a,b,c,d*}

A simple modular aptamer-based sensor (aptasensor) platform was prepared by combining the merits of rapid and efficient preparation of self-assembled monolayer of cucurbit[7]uril (CB[7] SAM) and the strong and specific binding affinity of CB[7] to ferrocenemethylammonium (FA), as an ultrastable supramolecular linker, to immobilize aptamers on CB[7] SAM.

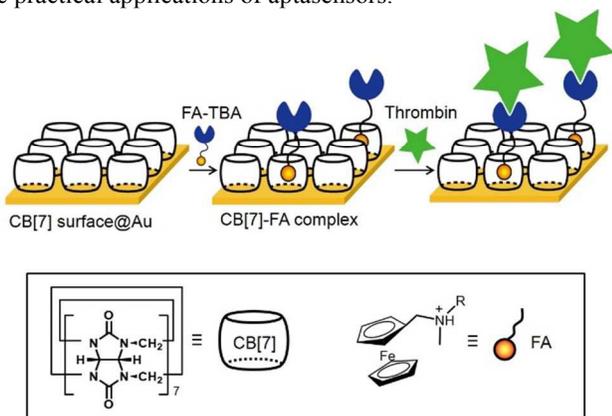
Aptamers are the artificial nucleotide sequences that fold into secondary and tertiary structures with high selectivity and specificity to target molecules including proteins, peptides and small organic molecules.¹ Along with these features, aptamers have shown extraordinary promise in analytical applications due to their easy chemical synthesis and modifications with reporter molecules, linkers, and other functional groups.² Many researchers have thus explored the aptamers as highly selective biorecognition components in bioassays and bio-sensing.²⁻³ Apart from the inherent advantages of biosensors, aptamer-based biosensors (aptasensors) offer the advantage of reusability utilizing reversible structural-switching.⁴ Furthermore, their small size and chemical functionalities allow efficient immobilization at high density,⁵ which is of vital importance in multiplexing miniaturized systems. In spite of these rapid advances, aptamer-based bioassays are still immature compared to antibody-based immunoassays, thus reflecting the limited availability of aptamer types and the relatively poor knowledge of surface-immobilization technologies for aptamers.⁶ In this regard, the multiple

processing steps for immobilization of aptamers on surfaces is a major concern to develop such systems.⁷ Streptavidin-biotin binding pair⁸ has extensively been used as an efficient linker system to immobilize aptamers on surfaces^{5b,9} because of their strong and specific noncovalent interactions (binding constant $\sim 10^{13} \text{ M}^{-1}$). The multiple processing steps include the pre-treatment of additional chemical reagents such as alkane thiols, functionalized dextrans, polyethyleneglycol thiols (PEG-SH) and also requires a chemical modification of proteins with delicate handling.^{7,10} Each step in this process claims to be a crucial parameter to the analytical performance of aptasensors. Such limitations therefore necessitate a new aptasensor platform that can simplify the processing steps using chemically stable binding pair which could be convenient in terms of storage and handling.

Earlier, we and other researchers reported that cucurbit[7]uril (CB[7]), a member of the host family cucurbit[*n*]uril (CB[*n*], *n* = 5-8, 10, 14)^{11,12} with a hydrophobic cavity and two identical carbonyl-fringed portals, binds ferrocenemethylammonium (FA) ion with an exceptionally high binding affinity with a binding constant of $\sim 10^{12} \text{ M}^{-1}$ in aqueous solution.¹³ This exceptionally strong binding affinity of CB[7] to FA led us to develop a synthetic system successfully employed, as an alternative artificial system to streptavidin-biotin binding pair, in biological applications such as immobilization of biomolecules on solid surface and membrane protein isolation.¹⁴ Recently, Li and others reported a method for the formation of self-assembled monolayer of CB[7] on a

gold surface by dipping a gold surface into a solution of CB[7].¹⁵ It was observed that CB[7] molecules on the gold surface are uniform in their orientation and hold carbonyl portals perpendicular to the plane of the surface to maintain the guest binding and recognition properties of CB[7]. Further, Brunsveld *et al.* demonstrated simple and reversible immobilization of FA-conjugated yellow fluorescent protein (FA-YFP) on CB[7].¹⁶ Although aptamers have become increasingly important molecular tools in the biosensor field because of the inherent advantages as mentioned above, the immobilization of aptamers on CB[7] has not been reported yet.

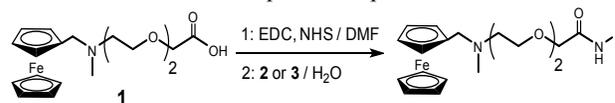
Described herein is a simple modular aptasensor platform by combining the merits of both simple preparation of CB[7] SAM and highly strong and selective binding affinity of CB[7] to FA. We utilize FA-CB[7] binding pair system as an ultrastable supramolecular linker, with the help of which various FA-conjugated aptamers can efficiently be immobilized on CB[7] SAM for surface plasmon resonance (SPR) studies. (Scheme 1) Our present aptasensor system bears unique features including 1) easy preparation of CB[7] SAM by either direct injection of CB[7] to a gold surface or dipping a gold surface into a solution of CB[7], 2) versatility of CB[7] SAM for immobilization of various FA-conjugated aptamers, 3) resistance to harsh conditions such as elevated temperature or unwanted enzymatic degradation, and 4) long term durability due to the usage of stable synthetic molecules,^{14b} instead of (strept)avidin proteins used in conventional aptasensors. This FA-CB[7] linker-based aptasensor may act as a simple and efficient bio-sensor including that for proteins and thus widen the practical applications of aptasensors.



Scheme 1. Immobilization of ferrocenemethylammonium-conjugated thrombin binding aptamer (FA-TBA) on a CB[7] SAM using the strong binding affinity of CB[7] to FA, and its application as an aptasensor chip for sensing thrombin.

CB[7] SAM was prepared by simply dipping a gold surface into a solution of CB[7] (1.0 mM in deionized H₂O) for 6 hr as per the literature reports^{15a} and conveniently kept at RT until being ready to use as a sensor chip. Direct injections of CB[7] solution (1.0 mM in deionized H₂O, 20 min, 20 μ L/min) to a gold surface mounted in a SPR machine were also conducted as an alternative method. After 5 sequential injections, we

observed approximately 500 RU (1000 RU = 1 ng/mm²)¹⁷ of sensor response (Fig. S1, ESI[†]) which turned out to be 0.43 pmol of CB[7] on 1 mm². This result suggested that ca. 55% of a plain gold surface is covered with CB[7] by considering the theoretical density of densely packed CB[7] (1.6 nm of diameter)^{12b} on a plane surface (0.75 pmol/mm²). In AFM images (Fig. S2, ESI[†]), small dots with approximately 1 nm in height and only few aggregation appeared on a gold surface after treatment of CB[7]. The SPR and AFM results suggested that even simple direct injections of CB[7] solution to a gold surface can lead to the formation of CB[7] SAM on a gold sensor chip. Since CB[7] is a stable molecule synthesized in a highly acidic condition at high temperature,^{11a, 11b} it has higher resistance to harsh conditions and better long term durability compared to (strept)avidin, which suggests that CB[7] SAM can be useful as a robust aptasensor platform.



Amine terminated thrombin DNA aptamer: 5'-NH₂C₆H₁₂-GGT TGG TGT GGT TGG-3' (2)
Amine terminated scrambled DNA sequences: 5'-NH₂C₆H₁₂-GGT GGT GGT TGT GGT-3' (3)

R= Thrombin DNA aptamer: -C₆H₁₂-GGT TGG TGT GGT TGG-3' (FA-TBA)
Scrambled DNA sequences: -C₆H₁₂-GGT GGT GGT TGT GGT-3' (FA-SDNA)

Scheme 2. Conjugation of ferrocenemethylammonium to thrombin binding aptamer (FA-TBA) and scrambled DNA sequences (FA-SDNA).

In the present work, a carefully studied aptamer (TBA, 15-mer DNA-based) that binds strongly and selectively to thrombin was chosen as a model probe.¹⁸ By exploiting FA-CB[7] interactions for the immobilization of TBA on CB[7] SAM, it was first "ferrocenylated"^{14a} by conjugation of carboxylated FA (1)^{14b} to aminoethyl group at 5' terminal using EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) coupling (Scheme 2). Thus formed ferrocenylated TBA (FA-TBA) was further purified and characterized by HPLC and ESI-mass spectroscopy, respectively (Fig. S4, ESI[†]). A binding constant of chemically modified TBA ($\sim 10^9$ M⁻¹) measured by filter binding assay¹⁹ was noted to be comparable to that of unmodified TBA. This implies that the ferrocenylation barely affects the binding affinity of TBA to its target protein, thrombin.²⁰

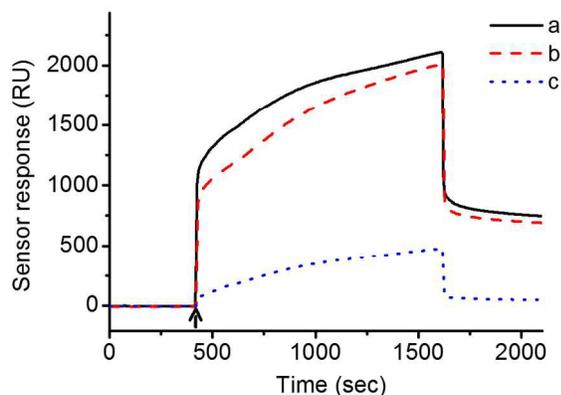


Figure 1. SPR sensorgrams of CB[7] SAM treated with a) FA-TBA, b) FA-SDNA and c) TBA (The arrow indicates injection of samples)

After 25 min of injection of FA-TBA and TBA to each channel on the CB[7] sensor chip, ~ 700 RU and 30 RU, respectively, were observed at 1920 sec (Fig. 1). The higher RU value (~ 23 -fold) for FA-TBA compared to TBA proves its importance for effective immobilization of aptamers. The converted density of FA-TBA on CB[7] SAM from the RU value (0.12 pmol/mm^2), suggested that approximately one out of four CB[7] molecules on average seemed to capture one FA-TBA. This result suggested successful immobilization of FA-TBA on CB[7] SAM by making the use of strong and specific interactions between CB[7] and FA. To check the versatility of CB[7] SAM for immobilization of various aptamers, we prepared FA-scrambled DNA (FA-SDNA) with the same molecular weight as FA-TBA but with different DNA sequences. (Scheme 2) The observed RU value i.e. 670, for FA-SDNA injection was almost in line with that of FA-TBA. This finding clearly shows that CB[7] SAM not only can immobilize FA-TBA but also other various aptamers as long as they are conjugated with FA utilizing strong and specific interaction between CB[7] and FA as a supramolecular linker.

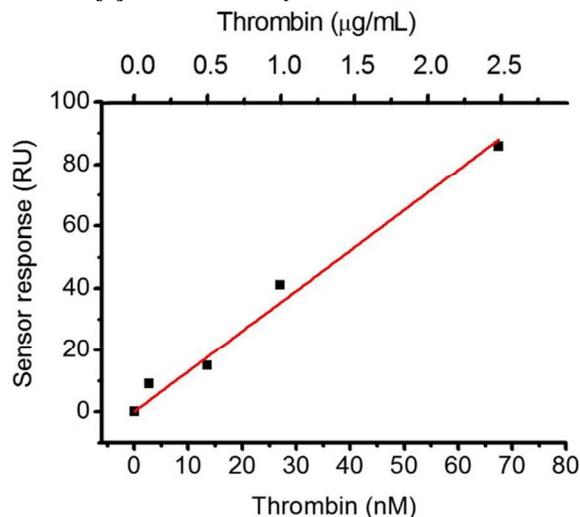


Figure 2. Sensor responses as a function of thrombin concentration on CB[7] SAM SPR sensor chip (15 min, $5 \mu\text{L/min}$)

Having established FA-aptamer@CB[7] SAM as a simple and modular aptasensor system, we examined the feasibility of FA-TBA@CB[7] SAM as a model SPR sensor chip. To confirm the selective recognition of thrombin, we injected it and bovine serum albumin (BSA, as a negative control) to a different channel on FA-TBA@CB[7] sensor chip. Accordingly, after 15 min of injection, we observed 340 RU for thrombin and 35 RU for BSA (Fig. S5, ESI†). These values signify the specific recognition and non-specific adsorption, respectively. Moreover, the higher sensor response of thrombin as compared to BSA, by one order of magnitude, denotes its selective recognition. Furthermore, a concentration dependent sensor response was observed by injection of different thrombin concentrations to a channel of the FA-TBA@CB[7] sensor chip. These recorded responses ranged from sub-ppm to ppm level, thus showing the sensing of target protein in nanomolar concentrations. Even though this noted analytical performance of FA-TBA@CB[7] sensor chip is comparable to that of streptavidin-biotin binding pair based system reported earlier,⁷ the former yet comes up with the benefits of effortless preparation, resistance to harsh conditions, long term durability and reusability by utilizing either reversible structural-switching of aptamers⁴ or reversible noncovalent binding property of CB[7] to FA^{12g, 21} as previously demonstrated with proteins.¹⁶

In summary, we successfully demonstrated the efficient and versatile immobilization of FA-conjugated aptamers with FA-TBA and FA-SDNA on CB[7] SAM by taking advantage of its facile formation on gold surface and also ultrastable binding of CB[7] to FA. The profound analysis of a target protein using FA-TBA@CB[7] SAM proved the utility of FA-CB[7] binding pair as an efficient linker system providing a new aptasensor platform. Furthermore, the optimization on formation of CB[7] SAM and immobilization of FA-aptamers using functionalized CB[7],²² which can further control the density of CB[7] on a gold surface, may result in the enhanced analytical performance of CB[7]-based aptasensors.

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Notes and references

^aCenter for Self-assembly and Complexity, Institute for Basic Science (IBS), Pohang, 790-784, Republic of Korea.

^bSchool of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, Pohang, 790-784, Republic of Korea.

^cDepartment of Chemistry, Pohang University of Science and Technology, Pohang, 790-784, Republic of Korea.

^dDivision of Advanced Materials Science, Pohang University of Science and Technology, Pohang, 790-784, Republic of Korea.

^eDivision of molecular and Life Science, Department of Life Science, Pohang University of Science and Technology, Pohang, 790-784, Republic of Korea

*These authors contributed equally to this work

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