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Cell Surface-Anchored Fluorescent Aptamer Sensor Enables Imaging of Chemical Transmitter Dynamics

Takeshi Tokunaga,[†,#] Shigeyuki Namiki,[‡,#] Katsuhiro Yamada,[†] Takahiro Imaishi,[†] Hiroshi Nonaka,[†] Kenzo Hirose,[‡] and Shinsuke Sando*[,†,§]

[†]INAMORI Frontier Research Center, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan
[‡]Department of Neurobiology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan
[§]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

Supporting Information

ABSTRACT: A fluorescent aptamer sensor was applied to the analysis of extracellular chemical transmitter dynamics. We utilized a tocopherol-labeled aptamer, which allowed the direct anchoring of the fluorescent aptamer on the cell surface while retaining its performance as a fluorescent sensor. The fast-responsive fluorescent DNA aptamer sensor, which targets adenine compounds, was anchored on the surface of brain astrocytes. Fluorescence imaging of the aptamer-anchored astrocytes enabled the real-time monitoring of release of adenine compounds as a gliotransmitter, which was synchronized with calcium wave propagation in neighboring cells.

Aptamers are functional oligonucleotides that work as *Host* molecules to specifically recognize a variety of *Guest* molecules: metal ions, small molecules, proteins, or cells.[1] Because of their high affinity and selectivity, there is much interest in applying aptamer technology to sensors.[2] In particular, the fluorescent aptamer sensor is one of the most attractive choices because the fluorescence readout can be recorded in real time and in situ, with a high spatiotemporal resolution. However, despite many efforts, practical cellular applications have been limited to only a few examples aimed at the intracellular analysis of target biomolecules.[3]

Karp et al. recently reported on a new application of fluorescent aptamer sensor.[4] They demonstrated that a structure-switching fluorescent aptamer covalently conjugated on membrane protein functioned as a sensor for probing cellular environments, for example, extracellular proteins. Independently, we have used a fluorescent aptamer sensor for sensing extracellular messenger molecules such as chemical transmitters. Here, we report on our approach for direct anchoring of a fluorescent aptamer sensor on the cell membrane, and we, for the first time, demonstrate that a fast-responsive aptamer sensor, anchored on cell surface, enables a real-time imaging of chemical transmitter dynamics.

Our target molecule in this work is a chemical transmitter released from glia cells in the brain. The glia cells are abundant in the brain and were once considered passive bystanders, but are now considered an essential cell in the regulation of synaptic transmission in neuronal networks.[5] The astrocyte is one of the major types of glia cells. The astrocyte forms a tripartite synapse through which it monitors neuronal synaptic activity and releases chemical transmitters, termed *gliotransmitters*, which control synaptic activity or coordinate the function of neighboring astrocytes (Figure 1).

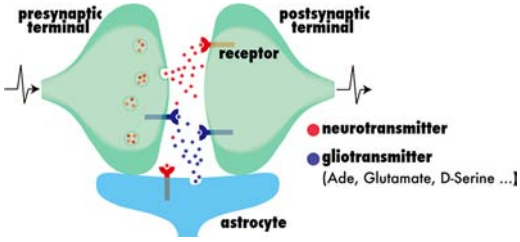

Figure 1. How astrocytes work to regulate synaptic activity.

A major gliotransmitter is adenine compound (Ade), mainly in the form of adenosine triphosphate (ATP). Recent research suggests that astrocytes release Ade as a main gliotransmitter via the multiple pathways including exocytosis.[6−8] Such gliotransmission is intrinsically involved in the control of the neuronal network and higher brain function. A tool for real-time monitoring of extracellular gliotransmitters is required for neurochemical studies. Although the luciferin−luciferase assay is a typical choice for analysis of extracellular ATP,[7,8] this approach has important disadvantages in temporal resolution because of the low off-rate of the light-emitting complex;[9] thus, the details of Ade dynamics remain unclear.

We have chosen a fluorescent aptamer sensor for real-time monitoring of extracellular Ade. An Ade-binding aptamer has been isolated[10] and Ade-targeting fluorescent aptamer sensors have been designed.[2] Therefore, Ade is an appropriate target for imaging using fluorescent aptamers. However, there are two important issues to be solved for this purpose.

The first issue is a quick response time. Because the neurological event is a fast biological phenomenon, typically in an order of seconds or milliseconds, the response of the fluorescent aptamer should be fast enough to allow real-time monitoring. We focused on the simple fluorescent aptamer sensor fApt, shown in Figure 2.[11] fApt is an Ade-binding DNA aptamer labeled simply with fluorescein (**f** in Figure 2) in proximity to the site of the binding pocket whose fluorescence

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fApt 5' ACCTGGG-**f**-GGAGTATTGCGGAGGAAGGT 3'

bio-fApt 5' Biotin-ACCTGGG-**f**-GGAGTATTGCGGAGGAAGGT 3'

toc-fApt 5' Tocopherol-ACCTGGG-**f**-GGAGTATTGCGGAGGAAGGT 3'

toc-frdmODN 5' Tocopherol-NNNNNNN-**f**-NNNNNNNNNNNNNNNNNNN 3'

f :

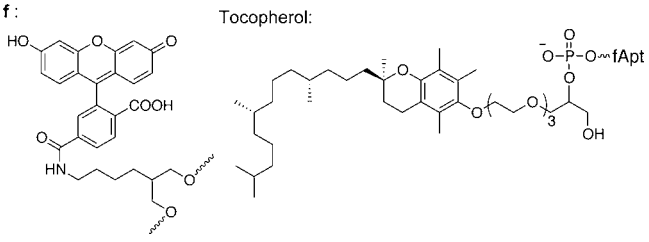

Figure 2. Fluorescent aptamers used in this study.

intensity increases upon binding of Ade. Because this simple fApt requires no large conformational change for Ade binding-induced fluorescence enhancement, the response time seemed much faster than typical structure-switching fluorescent aptamer sensors.

Our experiments showed this was the case. Stopped-flow fluorescence studies showed that the k_{on} and k_{off} values of the fApt–ATP interaction were 6.6×10^5 M^{-1} s^{-1} and 7.3×10 s^{-1}, respectively (Figure S1). This k_{off} value is far faster than that of luciferin-luciferase reaction (an order of 10^{-2} s^{-1}).[9] Also, these values are similar to those of SuperGluSnFR ($k_{on} = 3.0 \times 10^7$ M^{-1} s^{-1} and $k_{off} = 7.5 \times 10$ s^{-1}), a glutamate-targeting fluorescent reporter protein used in optical neurological imaging,[12] suggesting that the fApt would be effective for analyzing fast neurological events.

The second issue, when applying the potent fApt to extracellular molecular analysis, is how to localize the aptamer sensor on the extracellular domain, preferably on the cell surface, while retaining the sensor's performance and without damaging cellular activity.[7c]

As a first choice, we tried covalent conjugation of an amino functional group on cell membrane proteins. This method has been used successfully to immobilize protein-based[13] or aptamer-based[4] fluorescent sensors. Briefly, an amino group on the cell membrane was chemically modified with NHS-PEG_4-Biotin by amide bond formation, and then 5′-biotinylated bio-fApt (Figure 2) was attached on the cell surface via biotin–avidin complexation (Figure 3a). Confocal fluorescence imaging of bio-fApt-immobilized HeLa cells (bio-fApt-HeLa) showed that fluorescence was observed only at the cell membrane and that the bio-fApt sensor was immobilized on cell membrane by this procedure (Figure S2).

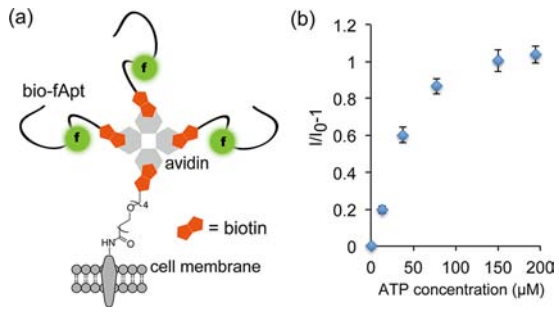

Figure 3. (a) Immobilization of the aptamer on the cell surface using covalent conjugation at an amino group. (b) ATP-dependent fluorescence enhancement of bio-fApt-HeLa cells. Error bars show standard deviations ($n = 6$).

We then evaluated the response of the immobilized bio-fApt to ATP. Addition of ATP to bio-fApt-HeLa cell culture solution resulted in a clear increase in fluorescence intensity. The change in fluorescence of bio-fApt-HeLa cells was dependent on the ATP concentration (Figure 3b), reaching $I/I_0 - 1 = 1.04$ (I = fluorescence intensity) in the presence of ~200 μM ATP. Although one fApt recognizes two Ades, it was shown that only second-Ade binding contributes to an increase in fluorescence of fApt.[11] In addition, an amount of fApt on cell surface should be much lower than that of target Ade, so we hypothesized that an influence of the first-Ade binding can be ignored. Under these conditions, the Ade-sensing with fApt on cell surface could be roughly assumed as a pseudo one-to-one model. In fact, a simple one-to-one binding curve fitted well to the observed fluorescence increase with $R^2 > 0.985$. From the curve fitting, the apparent dissociation constant ($K_d \pm$ SE) of bio-fApt and ATP interaction on bio-fApt-HeLa was determined as 45 ± 9 μM ($R^2 = 0.986$), which was almost similar to that in the buffer solution[14] (Figure S3a and ref 11). The observed apparent K_d value on the cell surface was within a good range for monitoring of ATP released from astrocytes, judging from the estimated ATP concentration of 78 μM at the releasing site.[8] These results proved that fApt can be immobilized on the cell surface while retaining its activity as a fluorescence sensor. Thus, the cell surface fApt satisfies the prerequisites for monitoring of Ade release.

As demonstrated above, the covalent conjugation method worked well. However, the procedure requires multiple processes. We then focused on a simpler approach, the direct anchoring of the fluorescent aptamer sensor by harnessing the power of a cell membrane-binding molecule.[15,16] We prepared a new aptamer toc-fApt having tocopherol at the 5′-end (Figure 2). Tocopherol (vitamin E) is a nontoxic lipophilic molecule that adheres directly to cellular membrane lipids. We expected that toc-fApt would attach to cells simply after its addition into cell cultures without chemical preactivation of the cell surface (Figure 4a).

We first checked the performance of this approach using HeLa cells. Confocal fluorescence imaging showed that toc-fApt was anchored on the cell membrane of HeLa cells (toc-fApt-HeLa, top-left in Figure 4b and S4) and did not induce toxicity under our experimental conditions (MTT assay in

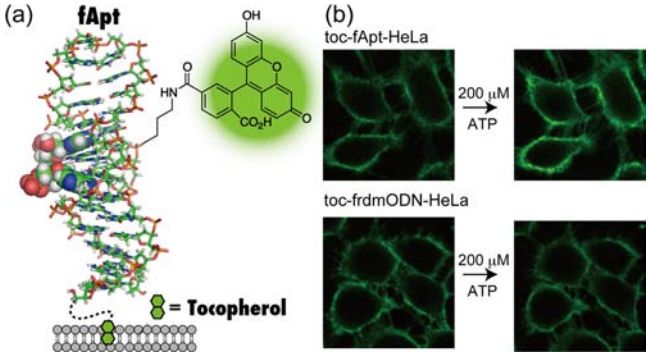

Figure 4. (a) Direct anchoring of toc-fApt on the cell surface. Structure of DNA aptamer was from PDB 1AW4. AMPs are shown in a sphere as a model of Ade. (b) Confocal fluorescence images of toc-fApt-HeLa (top) and toc-frdmODN-HeLa (bottom) in the absence (left) or presence (right) of 200 μM ATP.

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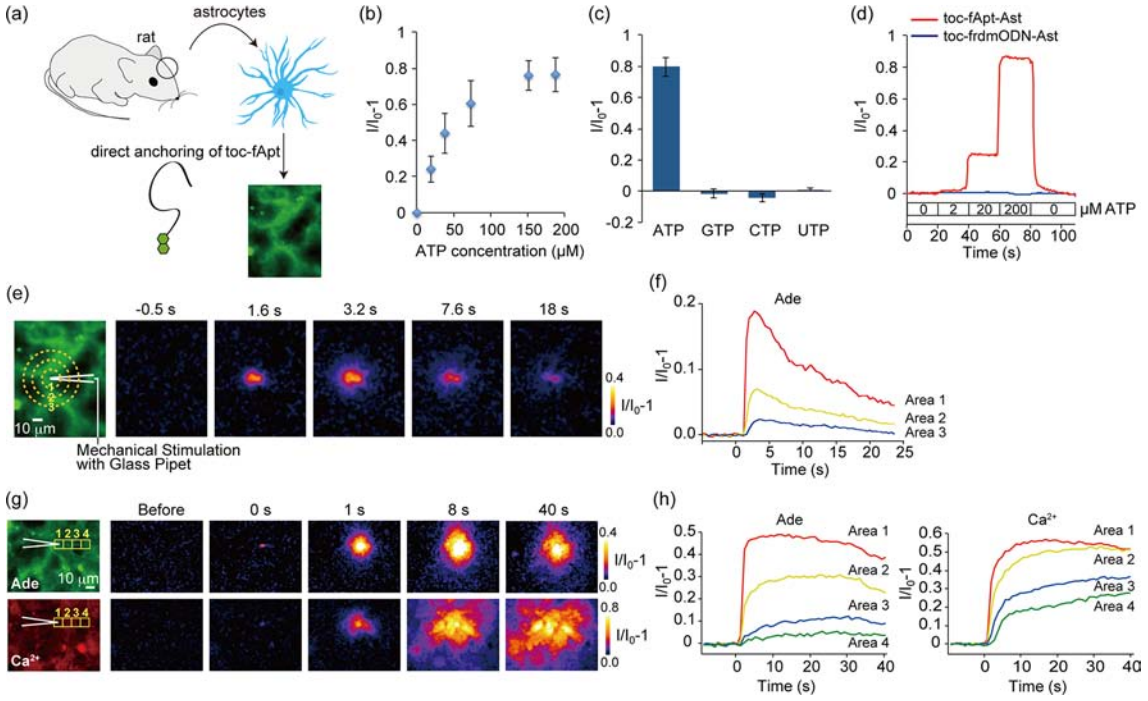

Figure 5. (a) Experimental procedures. (b) ATP-dependent fluorescence enhancement of toc-fApt-Ast cells. (c) Fluorescence enhancement of toc-fApt-Ast cells in the presence of nucleotide triphosphates (200 μM each). Error bars in (b) and (c) show standard deviations (n = 4). (d) Change in fluorescence of toc-fApt-Ast cells upon quick exchange of ATP concentration. (e) Time-lapse images of fluorescence enhancement of toc-fApt-Ast cells after gentle mechanical stimulation. Fluorescence monitoring conditions were set to fApt. (f) Time course of fluorescence enhancement ($I/I_0 - 1$) at the areas indicated by circles 1−3 in (e). (g) Time-lapse images of fluorescence enhancement of toc-fApt-Ast cells after mechanical stimulation. Fluorescence monitoring conditions were set to fApt (top) and the calcium indicator X-Rhod1-AM (bottom). (h) Time course of fluorescence enhancement ($I/I_0 - 1$) at the areas indicated by squares 1−4 in (g).

Figure S5). Importantly, the fluorescence intensity on the cell surface of toc-fApt-HeLa increased after addition of ATP (top-right in Figure 4b). By contrast, performing the same experiment using a random oligonucleotide having tocopherol and fluorescein (toc-frdmODN in Figure 2) produced nearly constant fluorescence intensity after addition of ATP (bottom in Figure 4b). This showed that the direct anchoring approach enabled the attachment of the fluorescent aptamer sensor while retaining its performance and without damaging cellular activity.

We then applied the direct anchoring method to brain astrocytes obtained from rats. The toc-fApt was anchored on the astrocyte cells (toc-fApt-Ast), and toc-fApt-Ast was subjected to fluorescence imaging (Figure 5a).

toc-fApt anchored on astrocytes worked as a fluorescence-on sensor that targeted Ade. The fluorescence intensity of toc-fApt-Ast responded to an increase in ATP concentration (external addition) with an apparent K_d of 46 ± 6 μM (R^2 = 0.992, Figure 5b). In addition, toc-fApt on astrocytes retained its original substrate selectivity. Addition of other nucleotide triphosphates (200 μM each) to the toc-fApt-Ast cell culture produced almost no increase in fluorescence (Figure 5c). The binding affinity and substrate selectivity on astrocytes were almost identical to those in solution (Figure S3). toc-fApt-Ast exhibited a quick fluorescence response. toc-fApt-Ast responded to the sudden increase or decrease in ATP concentration in cell culture, at least within a few seconds (red line in Figure 5d), whereas toc-frdmODN-Ast showed no fluorescence change (blue line in Figure 5d).

Having a promising fluorescent aptamer anchoring method in hand, we applied fApt for analysis of chemical transmitter release from brain astrocytes. Because gentle stimulation induces Ade (mostly ATP) release from astrocytes,[7,8] mechanical stimulation was applied to toc-fApt-Ast by gently touching the cells with a glass pipet using micromanipulators.

toc-fApt-Ast achieved real-time monitoring of Ade release from astrocytes. As shown clearly in the time-lapse fluorescence images (Figure 5e; see also Supporting Information movie 1), an increase in fluorescence of toc-fApt-Ast was observed around the stimulation point.[17]

Figure 5f shows the time course of the change in fluorescence ($I/I_0 - 1$) in the areas indicated by yellow circles 1−3 in Figure 5e. In all areas, the fluorescence intensity increased quickly and decreased slowly, an observation that is similar to previous extracellular ATP analyses by luciferin−luciferase assay,[7,8] supporting the idea that the observed fluorescence response corresponded to the Ade (ATP) release from astrocytes.

To investigate the influence of the Ade release on neighboring cellular activities, we then observed intercellular calcium dynamics simultaneously with Ade release. toc-fApt-Ast was loaded with a known calcium indicator X-Rhod1-AM, which has different excitation and emission wavelengths from those of fluorescein in fApt, and we imaged the effects using dual-wavelength fluorescence microscopy (Figure 5g and Supporting Information movie 2). In this experiment, to induce a longer Ade release, toc-fApt-Ast was subjected to slightly stronger mechanical stimulation. Upon stimulation, calcium propagation was ignited from the stimulation point (bottom line) in good synchronization with Ade release (top line). The observed continuous Ade release was consistent with the time scale of calcium wave appearance in the neighboring cells,[8] where the calcium wave continued for tens of seconds

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after astrocyte stimulation (Figure 5h). Extracellular Ade (ATP) can increase cellular calcium concentration.[7,8] Therefore, the observed synchronization confirms that the stimulation-induced change in fluorescence of toc-fApt on astrocytes responded to the Ade release.[18] This experiment clearly indicates the practical utility of the cell surface-anchored fluorescent aptamer sensor in the analysis of extracellular chemical transmitter dynamics.

In a neurological sense, the observed fluorescence responses are intriguing. Brain function is a very quick phenomenon, and typical neurotransmitters work in the order of milliseconds.[12,13] However, in this case, an interval of tens of seconds was required for the disappearance of Ade. As shown in Figure 5d, the dissociation kinetics of Ade from the fApt sensor was fast and responded to the change in ATP concentration within a few seconds. These results suggest that the observed slow decrease in fluorescence reflects a real cellular process and not an artifact derived from an inappropriate response time of the fluorescent aptamer sensor. Although further experiments are needed, it is reasonable to think that this slow Ade release ignites neuronal activity over a relatively long time scale.

In conclusion, we demonstrated the successful application of a fluorescent aptamer sensor in the analysis of Ade as extracellular chemical transmitter. The present strategy: (1) provided a simple immobilization of a fluorescent aptamer sensor on a living cell surface while retaining its performance (affinity, selectivity, fast response) and without damaging cellular activity; (2) achieved the fluorescence imaging of gliotransmitter release from brain astrocytes; and (3) revealed the potent slow release processes of a gliotransmitter that was synchronized with calcium wave propagation in neighboring cells. To our knowledge, this is the first report demonstrating that a fast-responsive fluorescent aptamer sensor, anchored on cell surface, can be a practical tool for real-time imaging of chemical transmitter dynamics.

From a more general point of view, the present research expanded the research field of aptamer sensors. Because various fluorescent aptamer sensors, targeting different molecules with diverse photochemical properties, can be designed and easily tuned in combination with a powerful SELEX technique and chemistry-based fluorescence-signaling approach as demonstrated so far, many other extracellular molecules can be targeted for analysis. At this stage, the fApt recognizes almost all types of Ade (Figure S3a). A detailed fluorescence imaging of Ade using a set of multicolor fluorescent aptamers that can discriminate types of Ade such as AMP, ADP, or ATP could also be a challenge. The present system allows only a relative quantification. However, absolute quantification is possible if this aptamer sensor is improved to be a ratiometric sensor by addition of another fluorophore. Of course, application is not restricted to the neural research. The present approach can be applied to investigation of diverse extracellular biochemical phenomena.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S6, methods, and movies 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

ssando@ifrc.kyushu-u.ac.jp

Author Contributions

[#]T.T. and S.N. contributed equally.

Notes

The authors declare no competing financial interest.

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(17) Under our experimental conditions, 82% (18/22) of stimulated cells induced fluorescent enhancement. Fluorescence enhancement was reproduced for stimulation at least twice (n = 6).

(18) Fluorescence enhancement was observed successfully by a more physiological stimulus of toc-fApt-Ast (mouse), hypotonic stress and thrombin addition, known to induce Ade release (Figure S6).