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Aptamer-Based Cell Imaging Reagents Capable of Fluorescence Switching

Yun Kyung Jung^{a,b}, Min-Ah Woo^a, H. Tom Soh^c, and Hyun Gyu Park^a

^aDepartment of Chemical and Biomolecular Engineering (BK21+ Program), KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea

^bSchool of Natural Science, UNIST, UNIST-gil 50, Eonyang-eup, Ulju-gun, Ulsan 689-798, Republic of Korea

^cMaterials Department, Department of Mechanical Engineering, University of California, Santa Barbara, Santa Barbara, CA 93106, USA

Abstract

We describe an aptamer-conjugated polydiacetylene imaging probe (ACP) that shows highly specific fluorescence switching upon binding to epithelial cancer cells that overexpress the tumor biomarker protein EpCAM (epithelial cell adhesion molecule) on their surface.

Fluorescence imaging of tissue and cell surface markers is a widely used technique in many areas of biology and medicine.^{1–5} Typically, such imaging is achieved with fluorophores conjugated to an affinity reagent (*e.g.* monoclonal antibody, peptide or aptamer) that specifically binds to the surface marker of interest.² In order to achieve high-contrast imaging, it is imperative that the affinity reagent binds specifically to the surface marker with high affinity, and that the fluorophore is bright and photostable. Ideally, the fluorophore should exhibit low background fluorescence when not specifically bound to the surface marker, such that a visible signal is only generated in the bound state. Unfortunately, this is a major challenge for most fluorophores, for which the fluorescence intensity and wavelength remain constant regardless of their binding state.

Active probes capable of changing their fluorescence state – for example, switching from a “dark state” when unbound to a “bright state” when specifically bound to their target – offer a means for obtaining dramatically improved imaging contrast. Recent years have witnessed a growing interest in “switchable” fluorescence reporters such as Dronpa,³ cyanine dyes,⁴ and polydiacetylenes (PDA).⁵ In particular, PDAs – a family of nanoscale, conjugated polymers synthesized by polymerizing monomeric diacetylene lipids through UV irradiation – offer many advantages, because they are photostable, exhibit low cytotoxicity, and can be readily conjugated to a wide range of biopolymers.⁵ A number of recent publications have reported PDAs capable of switching their fluorescence state in response to various external stimuli. For example, Kim *et al.* developed micropatterned PDAs that undergo fluorogenic transitions upon thermal stress or interaction with cyclodextrin.^{5a,5b} Our group showed that

the fluorescence of streptavidin-functionalized PDAs can be modulated upon binding to biotinylated DNAs.^{5c} Importantly, Jelinek *et al.* have reported that the fluorescence of PDA attached onto live cell surfaces changes when it non-specifically interacts with cell membrane-perturbing molecules.^{5d-f} Although these pioneering examples have blazed a promising trail, maximizing the utility of PDA probes will require a novel class of probes that do not respond when they non-specifically interact with cell surfaces, and only switch their fluorescence state when they bind to specific cell surface makers. To the best of our knowledge, such PDA probes have not yet been demonstrated.

Toward this end, here we describe an active PDA imaging probe that switches its fluorescence state when it binds to a specific cell-surface marker. Our probe is functionalized with a high-affinity DNA aptamer that has been selected for specific binding to a cell-surface marker of interest. Our aptamer-conjugated PDA (ACP) probe is prepared by incorporating aptamer-conjugated diacetylene monomers into a diacetylenic lipid matrix comprising dimyristoyl phosphatidylethanolamine (DMPE) and 10,12-pentacosadiynoic acid (PCDA).⁶ In the unbound state, our ACP stays “dark” in the red-channel because the conjugated (ene-yne) polymer backbone remains unchanged (no emission peak) (Scheme 1, top). In contrast, when the ACP binds to its target surface marker, it switches to a “bright” state in the red-channel (emission peak at 563 nm) because the conjugated backbone of PDA undergoes a conformational transition that causes a red-shift in its fluorescence (Scheme 1, bottom). As a model target, we chose the epithelial cell adhesion molecule (EpCAM) protein; EpCAM is a tumor-specific antigen for malignancies of epithelial lineage, and is used as a marker for circulating tumor cells (CTCs).⁷ We show that our ACP probes enable specific visualization of epithelial cancer cells by switching their fluorescence emission from nonfluorescence (blue state) to fluorescence ($\lambda_{em} = 563$ nm, red state) upon binding the EpCAM molecules present on the cell surface.

Although RNA and DNA aptamers for EpCAM have been previously reported,⁸ we selected our own DNA aptamers to achieve higher specificity by utilizing the microfluidic SELEX (M-SELEX) method previously described by our group (Scheme S1[†]).⁹ The efficiency of our SELEX process was verified after one round of positive selection (Fig. S1[†]). We performed three rounds of positive selection using EpCAM-coated magnetic beads and one round of negative selection using bovine serum albumin (BSA)-coated magnetic beads to obtain an enriched aptamer pool (see the ESI[†]). We then measured the binding affinity of the enriched pool for EpCAM via a fluorescence-based bead-binding assay.⁹ Assuming Langmuirian binding between EpCAM and the aptamers, we measured the average equilibrium dissociation constant (K_d) of the enriched pool to be 8.4 ± 2.2 nM (Fig. 1A, ●). These aptamers showed negligible binding to BSA-coated or uncoated carboxyl magnetic beads (Fig. 1A, ▲ and ■).

We obtained sequences for individual EpCAM aptamers by cloning the enriched aptamer pool into the TATA cloning vector and transforming into *E. coli* (see the ESI[†]). From the

[†]Electronic Supplementary Information (ESI) available: Experimental details, scheme of aptamer selection and conjugation with diacetylene monomer, EpCAM binding analysis after one round selection, K_d values of representative aptamer sequences for EpCAM, epithelial cancer cell imaging by **JYK-02** ACP probe, and SEM and DLS analysis of the **JYK-01** ACP probes after protein addition. See DOI: 10.1039/b000000x/

fifty randomly picked clones, we identified three clusters that showed notable sequence similarity. We selected representative sequences from each cluster and measured their K_d (see the ESI[†] for experimental details). Aptamers from all three clusters showed K_d in the low nanomolar range (Fig. S2[†]); the aptamer with the highest affinity (**JYK-01**; 5'-TGAAGGTTTCGTTGTTTCGGTGGGTGTAGACTCTTTAGAAGAGATACAGATTTTGGGAATG-3') exhibited a K_d of 8.6 ± 2.5 nM. The binding affinity of this aptamer was higher than previously described EpCAM aptamers⁸ and comparable to engineered anti-EpCAM antibodies¹⁰ (Fig. 1B).

We synthesized ACPs by modifying the 5' ends of our **JYK-01** aptamers with an amine group and conjugating them to diacetylene monomers (10,12-tricosadiynoic acid; TCDA) through EDC/NHS coupling chemistry (Scheme S1). In order to verify the functionality and specificity of our ACP probe, we measured the fluorescence after incubating the ACP with EpCAM in solution (Fig. 1C; excitation at 485 nm, emission at 563 nm).^{5e,5f} We simultaneously tested the specificity of our ACP probes by challenging them with two unrelated proteins, bovine serum albumin (BSA) and immunoglobulin G (IgG). Compared to the strong fluorescence signal from EpCAM, BSA and IgG produced negligible signal. To further confirm that the fluorescence change is indeed the result of specific binding between EpCAM and **JYK-01** aptamer, we fabricated an ACP containing a random sequence (5'-TAAGTATATCGTGCCTGCGACTATGTTATGATGAGGCAGTCTTTAACCTGACTCGTAATA-3'). The resulting ACP probes exhibited negligible fluorescence signal when challenged with EpCAM (Fig. 1C), adding further evidence that the change in fluorescence requires specific binding between **JYK-01** and EpCAM. To quantify the sensitivity of our ACP probe, we generated a calibration curve by plotting the change in fluorescence as a function of EpCAM concentration (Fig. 1D). We characterized the limit of detection (LOD) using the $3s_b/m$ criterion,¹¹ where s_b is the standard deviation of a negative control (blank) and m is the slope in the linear range. Using this metric, we estimated a LOD of ~ 1 ng/mL for our sensor.

We tested the capacity of the ACP probes to switch fluorescence upon binding to EpCAM on cell surfaces (Fig. 2). As target cells, we used the human colon adenocarcinoma cell line HT-29, which has an epithelial morphology and is known to express EpCAM.¹² We verified EpCAM expression by incubating these cells with an Alexa Fluor 555-labeled anti-EpCAM antibody, and observed strong fluorescence (Row 1, Left). We used two different cells as negative controls: normal human fibroblast cell line WI-38, derived from a non-tumorigenic epithelial lineage, and melanoma cell line WM-266-4, which originated from a different non-epithelial lineage. Both cell types showed negligible fluorescence when incubated with the same antibody (Row 1, Center and Right). In addition, we measured the fluorescence from ACPs conjugated to random DNA sequences as negative controls and did not observe any appreciable fluorescence signal for any of the three cell lines (Row 2). Critically, ACP probes conjugated to **JYK-01** aptamer specifically labeled HT-29 cells expressing EpCAM with strong fluorescence intensity comparable to that seen with the antibodies (Row 3, Left). The same ACP probes exhibited negligible fluorescence when incubated with either of the EpCAM-negative cell lines, WI-38 (Row 3, Center) or WM-266-4 (Row 3, Right).

Several recent studies have investigated the underlying mechanism behind the observed fluorescence switching,^{5b,5g–5j,13} and much of the evidence suggests that this switching is the result of mechanical stress on the PDA structure caused by binding to EpCAM. Such external stresses on the PDA structure are known to trigger electronic transitions in the delocalized π -electron networks of the PDA backbone and thereby change the optical properties. In this model, radiative transition (decay) from a higher excited Ag symmetry state (dipole-forbidden) to the lowest excited Bu state (dipole-allowed) is believed to be responsible for shifting the fluorescence wavelength from short (blue) to long (red).^{5b,5g–5j,13} We would therefore suppose that direct mechanical interaction between the ACP probes and EpCAM is a necessary requisite for fluorescence switching to occur.

To further support this model for fluorogenic transitions of ACP probes, we investigated changes in probe morphology and size before and after the addition of EpCAM via SEM and DLS.⁶ We observed that in the absence of EpCAM, ACP probes are monodisperse and spherical in shape with an approximate diameter of ~364 nm (Fig. S4a and S4d[†]). However, upon addition of EpCAM, the ACP probes aggregate and show a more dense interior structure, with sizes ranging up to ~933 nm (Fig. S4b and S4d[†]). Adding an unrelated protein (*i.e.*, IgG) does not trigger the same changes in size and morphology (Fig. S4c[†]). These results add further evidence that fluorescence switching is indeed caused by physical stress and morphological changes in the PDAs caused by binding to EpCAM on the cell surface.

Conclusions

In summary, we report an active aptamer-conjugated PDA probe that switches its fluorescence when specifically bound to a targeted cell-surface marker. We discovered DNA aptamers that bind to the EpCAM protein and used these to synthesize ACP probes that enable specific visualization of EpCAM-positive cells by switching their fluorescence emission wavelength. Given that aptamers are available for a broad range of surface markers, and PDA nanoparticles offer facile synthesis, low cytotoxicity,^{5d,5f} and excellent photostability,¹⁴ we believe that a deeper understanding of fluorescence-switching mechanism could lead to next generation of smart imaging probes with higher specificity, signal intensity and contrast.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(A) We used a fluorescence-based bead assay to measure the binding affinity of the enriched aptamer pool. After three rounds of positive selection with EpCAM and a single round of negative selection with BSA, aptamers showed nanomolar affinity for EpCAM with negligible binding to BSA-coated or uncoated beads. (B) We used the same assay to determine the binding affinity of the **JYK-01** aptamer. (C) Specificity analysis of **JYK-01**- or random sequence-functionalized ACPs for EpCAM protein. Each ACP was incubated for 1 hour with a protein-free control (Con) or 4 ng/mL of EpCAM or non-target proteins BSA and IgG. (D) Quantitative fluorescence detection of **JYK-01**-conjugated ACPs as a function of EpCAM concentration.

Fig. 2.

Epithelial cancer cell imaging, using ACP probes that target overexpressed EpCAM protein at the cell surface. Bright-field (left) and confocal fluorescence (right) microscopy images of HT-29 (epithelial cancer), WI-38 (normal epithelial; EpCAM-negative), and WM-266-4 (human melanoma; EpCAM-negative) cells. The images were taken after 2-hour incubation with (Row 1) Alexa Fluor 555-labeled anti-EpCAM antibody, (Row 2) ACP with a random aptamer, and (Row 3) ACP with **JYK-01**. Nuclei were labelled with DAPI (blue). Scale bar = 20 μm .

Scheme 1.

Epithelial cancer cell imaging utilizing fluorescent polydiacetylene (PDA) molecular probes conjugated to an EpCAM aptamer.