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Modulating Aptamer Specificity with pH-Responsive DNA Bonds

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Abstract

Aptamers that recognize specific cells in a complex environment have emerged as invaluable molecular tools in bioanalysis and in the development of targeted therapeutics. The selective recognition of aptamers, however, can be compromised by the coexistence of target receptors on both target cells and other cells. To address this problem, we constructed a structure-switchable aptamer (SW-Apt) with reconfigurable binding affinity in accordance with the microenvironment of target cells. The SW-Apt makes use of i-motifs, which are quadruplex structures that form in sequences rich in cytosine. More specifically, we report the design of single-stranded, pH-responsive i-motif-modified aptamers able to bind specifically with target cells by exploiting their pH. Here, the i-motif serves as a structural domain to either facilitate the binding ability of aptamers to target cells or suppress the binding ability of aptamers to nontarget cell based on the pH of the cellular microenvironment. SW-Apt exhibited high binding ability with target cells at acidic pH, while no obvious binding was observed at physiological pH. The i-motif-induced structure-switching was verified with Förster resonance energy transfer and circular dichroism spectroscopy. Notably, SW-Apt exhibits high specificity in serum and excellent stability, likely attributed to the folded quadruplex i-motif structure. This study provides a simple and efficient strategy to chemically modulate aptamer binding ability and thus improve aptamer binding specificity to target cells, irrespective of the coexistence of identical receptors on target and nontarget cells.

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Supporting Information

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Experimental details, lists of sequences studied, binding affinity analyses, folding/unfolding transition as a function of pH, kinetic traces, stability analysis, cytotoxicity study, and cell viability studies, including Table S1 and Figures S1–S9 (PDF)

Graphical Abstract

INTRODUCTION

Aptamers, often termed “chemical antibodies”, are high-affinity, single-stranded nucleic acid ligands that can specifically bind with a wide range of targets, from ions to proteins.^{1–6} Because aptamers exhibit many favorable features, such as small size, easy synthesis and modification, and low immunogenicity, they have attracted extensive attention for broad applications.^{2,3,5–7} However, development of molecular tools for targeting distinct cells in a complex environment constitutes one of the central challenges in molecular medicine. To meet this need, we recently reported a cell-based selection strategy (“cell-based systematic evolution of ligands by exponential enrichment”, or Cell-SELEX) to select aptamers as molecular probes for cancer diagnosis and cancer biomarker discovery.^{8,9} The selection process not only is fast and straightforward but also can be carried out without prior knowledge of proteins on the cell surfaces. The selected aptamers can bind with target cells with high affinity and selectivity compared to control cells, and our group has identified several such cell-targeting aptamers, including, for example, aptamer sgc8, our model aptamer, which binds to tyrosine kinase-7 (PTK7), a protein marker typically upregulated in cancer cells.^{8,10} While promising, aptamers, as recognition moieties, can be compromised by the coexistence of identical protein receptors on both target and nontarget cells. For example, most antigens targeted by antibodies and aptamers are not exclusively expressed on tumor cells. Instead, they are expressed at lower levels, or even at similar levels, in certain healthy tissues.^{11,12} It is therefore not surprising that toxicity toward healthy tissue cannot be avoided under these circumstances and that such toxicity has often been observed with targeted therapies.^{7,13} While research to improve targeting specificity has mainly focused on combinatorial approaches that can recognize two or more cell surface markers,^{14–17} smart aptamers capable of distinguishing a target cell receptor from the same receptor on the membranes of nontarget cells, but without the need to design a new recognition sequence, are in high demand.

In nature, binding specificity arises from the three-dimensional structures of substrates and enzymes. Carrying this idea forward in synthetic chemistry, we reasoned that the concept of structure-defined specificity could be grafted onto smart aptamers engineered to modulate specificity toward target and nontarget cells having the same surface receptors. A strategy that would improve selective targeting of receptors only on the target cell of interest would

be one that facilitates binding conformation of aptamers on target cells but suppresses such binding conformation on nontarget cells.

Recognizing stimuli present in the cellular microenvironment, recent studies have advanced the possibility of encoding molecular probes and therapeutics with stimuli-responsive properties, such as pH, for cell-specific imaging¹⁸ and biomedical applications.^{19,20} In particular, the i-motif is stabilized by acidic conditions. These structures consist of two parallel-stranded DNAs held together in an antiparallel orientation by intercalated, cytosine-cytosine⁺ base pairs.²¹ This switchable DNA structure has been intensively used in DNA nanotechnology for molecular sensing^{22,23} and therapeutic applications.^{24,25} So far, however, investigators have not applied i-motif to modulate aptamer specificity. Moreover, compared to antibodies and small peptides, an attractive advantage of aptamers is their ease of engineering and coupling with functional DNA nanostructures. Therefore, we explored the potential of i-motif-functionalized smart aptamers able to distinguish the same markers on different cells based on intracellular pH.

In this work, we report the design of an i-motif-modified aptamer and study its binding ability toward target cells in acidic and neutral microenvironments to modulate aptamer specificity (Scheme 1). As shown in Figure 1A, we added a split i-motif to aptamer sgc8 which, as noted above, recognizes PTK-7. The modified aptamer comprises three domains—recognition, linker, and modulator—hence the term split i-motif. Because i-motifs are unstructured at physiological pH, the linker region is disrupted, preventing recognition loops from folding and binding to receptors. However, at acidic pH, i-motifs fold, rendering a restricted orientation of the aptamer's recognition loop, thereby enabling the restoration of its targeting ability. We demonstrated that the designed SW-Apt could efficiently recognize target antigens in an acidic environment, but not the same antigen at physiological pH. The incorporation of the i-motif into aptamer sgc8 did not compromise the high binding affinity, while it allowed control over aptamer specificity according to microenvironmental stimulus, namely acidic vs physiological pH. This shows the potential of using i-motif-engineered aptamers to improve specificity toward target cells while eliminating the often-harmful targeting of nontarget cells.

RESULTS AND DISCUSSION

To engineer this smart aptamer, we first needed to identify an easily modifiable structural element. An interesting feature of aptamers is that they consist of 10–100 nucleotides, but only a few of these play a major role in target recognition and binding. Typically, these key residues alone have no function. It is only when the entire aptamer is folded into a distinct tertiary structure that a function emerges. Thus, one major role of the remaining nucleotides is precise control over the conformation of the aptamer and retention of key residues at the correct position. According to our previous report,²⁶ the loop and the first three base pairs near the loop of sgc8 are critical for binding. This was last exemplified by our circular bivalent aptamer which shows improved binding ability compared to the monovalent aptamer.^{27,28} When two aptamers are cyclized, the loop structure is not changed, but the stem is elongated, resulting in an increase of thermal stability, leading to enhanced binding affinity. Therefore, in this work, we carefully examined the effect of stem length on binding

ability of aptamers with targeted cells. Table S1 shows the DNA sequences used herein. CCRF-CEM cells were used as our model by the high expression of PTK7.⁸ Indeed, binding affinity significantly decreased along with decreasing length of aptamer stem from 7 to 3 base pairs (bp) (Figure S1A). To our surprise, increasing the stem length from 7 to 15 bp resulted in significantly higher affinity (Figure S1C). Therefore, we decided to focus our engineering efforts on the outer portion (5' and 3' ends) of the stem and use the i-motif as the outer stem of the aptamer to dynamically change the stem length and fine-tune aptamer binding ability in a complex environment.

Next, we asked how an i-motif could be incorporated into an aptamer to maximize switching efficiency and, in turn, increase binding affinity in accordance with microenvironmental stimulus. To accomplish this, we chose a reported i-motif with the required responsive pH range between 6.5 and 7.2 and midpoint pH of 6.9.²⁹ To illustrate the significance of stem length in maintaining loop conformation, we designed five SW-Apt's: i-motif-modified aptamer sgc8 with 3, 4, 5, 6, and 7 bp stem (isgc8-3, isgc8-4, isgc8-5, isgc8-6, and isgc8-7). A pH-insensitive rhodamine fluorescent dye (TAMRA) was used to label DNA probes to avoid false readout resulting from the low-pH quenching of certain fluorophores. We used CCRF-CEM cells in binding buffer at pH 7.3 and pH 6.5 to mimic normal PTK7-positive cells at physiological pH and tumor cells in an acidic tumor microenvironment. The binding affinities of the five aptamers were determined by treating cells with a range of concentrations at their corresponding pH values at 4 °C for 30 min. Interestingly, as shown in Figure 1B and Figure S2, modified aptamers with long linkers (e.g., isgc8-7, $K_d = 14.7 \pm 1.7$ nM at pH 6.5, $K_d = 78.1 \pm 29.8$ nM at pH 7.3, $K_d(\text{pH } 7.3)/K_d(\text{pH } 6.5) = 5.57$) showed better binding affinity, but exhibited smaller $K_d(\text{pH } 7.3)/K_d(\text{pH } 6.5)$ ratios compared to aptamers with short linkers (e.g., isgc8-4, $K_d = 150.6 \pm 71.1$ nM at pH 6.5, $K_d > 1000$ nM at pH 7.3, $K_d(\text{pH } 7.3)/K_d(\text{pH } 6.5) > 6.66$). The influence of pH on sgc8 binding was investigated. No significant difference in binding affinity of sgc8 (without modification) in the two different pH buffers was observed (Figure S3), suggesting that such small pH range had no effect on aptamer binding. Moreover, no significant enhancement of fluorescence intensity was observed for negative Ramos cells, confirming the binding specificity of the modified aptamers (Figure 2). Since the aim here was to generate an aptamer with good binding ability at acidic pH and low, or no, binding at neutral pH, isgc8-5 (Figure 1C) was selected because of its moderate binding affinity ($K_d = 52.9 \pm 1.5$ nM at pH 6.5) and best $K_d(\text{pH } 7.3)/K_d(\text{pH } 6.5)$ ratio (>18.9). Thus, isgc8-5, which has a moderate linker length, provides a compromise of the lower K_d with longer linker length and the larger pH 7.3/pH 6.5 ratio with shorter linker length.

Having demonstrated efficient modulation of aptamer binding affinity at different pH values, we next investigated the proposed mechanism of SW-Apt response to microenvironmental pH. To prove that the conformation of the i-motif-modified aptamer could be switched under the influence of microenvironmental pH, we first employed Förster resonance energy transfer (FRET) to reveal the formation of the i-motif structure at different pH levels. The isgc8-5 sequence was covalently labeled with a fluorophore (TAMRA) and a quencher (BHQ-2) at the 3' and 5' ends (Q-isgc8-5). In addition, we prepared control oligonucleotides with the same aptamer sequence and poly T having the same length, but without secondary structure (Q-Tsgc8-5). As shown in Figure 3A, the emission spectra of i-

motif-modified aptamer showed almost 10-fold increase of quenching at 580 nm, while the control aptamer did not show significant difference, indicating the occurrence of FRET and the formation of an i-motif closed architecture. Folding/unfolding transition of isgc8–5 were determined by monitoring the fluorescence signal at 584 nm with pH changing from 7.4 to 6.5 (Figure S4). The in vitro transformation of SW-Apt was characterized using Q-isgc8–5 by quickly changing pH from 6.5 to 7.3 and from 7.3 to 6.5. The probe could switch from a closed form to a random coil, and vice versa. As shown in Figure S5, the transformational rates of SW-Apt between the two states are moderately fast ($T_{1/2, \text{closing}} \approx 12$ s, $T_{1/2, \text{opening}} \approx 5$ s). The response times are not the same but in the same order of magnitude with a i-motif based DNA nanomachine.²² Because adding a hairpin-like aptamer may affect the structure and folding of the modified i-motif, we then confirmed the pH-induced structural folding and unfolding transitions of the isgc8–5 via circular dichroism (CD) spectroscopy. As shown in Figure 3B, at pH 6.5, the CD spectra showed a positive peak at 286 nm and a negative peak at 254 nm, which is the characteristic spectrum of an i-motif as reported in the literature,³⁰ indicating the formation of an i-motif structure. However, we observed only minimal change of the CD spectra of the control between the two pH values, again confirming that lowering the pH from 7.3 to 6.5 has almost no effect on the hairpin structure of the aptamer. Taken together, these results confirmed that i-motif serves as a structural domain capable of both facilitating and suppressing aptamer formation.

Encouraged by efficient control over aptamer binding and having confirmed its mechanism of action, we sought to determine if the switchable property of the i-motif-based SW-Apt could be maintained in biological media at physiological temperature. However, we first determined the effect of i-motif incorporation on stability in serum. Based on the remarkable stability of aptamer AS 1411 in serum by its compact G-quadruplex structure,³¹ we reasoned that the folded i-motif might also improve stability of the modified aptamer in serum. To address this, the stability of normal aptamer and SW-Apt in 10% fetal bovine serum (FBS) at pH 6.5 and 7.3 was tested at 37 °C. As shown in Figure 4A, sgc8 was unstable at both pH levels, their bands disappearing within 12 h. Bar graphs have been added in order to clearly and quantitatively compare the stabilities of isgc8–5 and sgc8 (Figure 4B,C). Notably, when isgc8–5 was incubated in 10% FBS, the intensity of the band dropped much more slowly. Especially, at pH 6.5, the band was still evident after 24 h incubation, indicating that i-motif had increased the stability of the hairpin aptamer in biological media, which is necessary for practical applications in targeted delivery. Stability analysis of i-motif only with 10% FBS at different pH values was tested and confirmed the enhanced stability of folded i-motif in serum-containing media (Figure S6).

Binding ability and specificity of the SW-Apt in serum-containing media were carried out in 10% FBS at two different pH values. As shown in Figure 5A, Lib could not bind to target cells at either pH 6.5 or pH 7.3 in 10% FBS, but sgc8 bound to the cells at both pH values. In contrast, isgc8–5 showed only minimal binding in 10% FBS at pH 7.3, but retained its high binding affinity in acidic media in good agreement with the tests in binding buffer. The target cells treated with isgc8–5 in acidic media exhibited much higher fluorescence on the surfaces than cells in neutral media, again confirming the efficient switchability of the target binding of the SW-Apt with antigens on the surfaces of living cells (Figure 5C). Moreover, when Ramos cells, a cell line with low PTK7 expression,⁸ were treated with isgc8–5, no

obvious fluorescence shift was observed (Figure 5B). Importantly, no obvious toxicity of isgc8-5 was observed, indicating high biocompatibility (Figure S7), along with modulated specificity, thereby demonstrating the suitability of SW-Apt for targeting of cell surface antigens in acidic microenvironments. We also studied this capability of SW-Apt in targeted therapeutic drug delivery by conjugating a porphyrin-based photosensitizer, chlorine e6 (Ce6) to 5' end of modified aptamer for photodynamic therapy (PDT). Various concentrations of the conjugates were exposed to CCRF-CEM and Ramos cells (Figure S8), and cell viability after treatment was determined by MTS assay. As shown in Figure 6, isgc8-5 can deliver Ce6 to induce cytotoxicity to CCRF-CEM in acidic media, while sgc8 did not exhibit a significant selectivity against CCRF-CEM cell at different pH values (Figure S9), further supporting modulated specificity of the SW-Apt.

CONCLUSION

We have successfully developed an allosterically regulated, highly pH-sensitive, binding switchable aptamer that can operate on cell surface antigens in a mimetic acidic tumor microenvironment, but not with the same antigens in neutral media. Compared to the normal aptamer, SW-Apt will be useful in solving the problem of identical target antigens on both tumor and healthy cells. Because of molecular engineering of a DNA quadruplex structure into a cancer-targeting aptamer, the hybrid DNA SW-Apt showed an enhanced stability in biological media, especially in acidic environments, without the use of any chemical modifications or nanomaterials, significantly improving *in vivo* applications. To apply this strategy to another hairpin aptamer, or quadruplex aptamer, a key challenge is how to identify the structural element, which can be discovered by structure–function relationship study or cocrystal structure analysis. If the structural domain is identified, i-motif or any other dynamic structure can be applied to replace it. To ensure that at a certain state aptamer functional structure can be restored and made functional again, careful calculation and optimization may be needed. For example, the distance and orientation of the rest nucleotides, if possible, should be taken into consideration. Additionally, by extending the selection of dynamic DNA structure responses to different stimuli, like light, temperature, or metal ions, we anticipate that the strategy demonstrated herein will be simple and efficient for the generation of smart molecular probes or ligands for various biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. (A) Structures of five SW-Apt's tested. (B) Results of binding affinity to CCRF-CEM cells at 4 °C in binding buffer (pH 6.5 and pH 7.3), as determined by flow cytometry. (C) Binding curves of isgc8-5 to CCRF-CEM at pH 6.5 (red) and pH 7.3 (black).

Figure 2.

Specificity analysis of the SW-Apt's. Binding affinity of the SW-Apt's to CCRF-CEM cells (left) and Ramos cells (right) characterized by flow cytometry. The concentrations of the probes were 100 nM. The incubation temperature was 4 °C.

Figure 3.

(A) Fluorescence spectra of Q-isgc8-5 (left) and Q-Tsgc8-5 (right) in the pH range from 6.5 to 7.4. All spectra were measured at $\lambda_{\text{ex}} = 535$ nm. (B) CD spectra of isgc8-5 (left) and Tsgc8-5 (right) at pH 6.5 and 7.3. The measurements were taken at room temperature.

Figure 4.

(A) Stability analysis of isgc8-5 and sgc8 after treatment with 10% FBS for different times and at different pH values, as determined by UREA PAGE. The concentrations of isgc8-5 and sgc8 were 200 nM. Quantification analysis of band intensities of isgc8-5 (B) and sgc8 (C).

Figure 5. Binding ability analysis of SW-Apt (isgc8-5) in biological media. Flow cytometric analysis of CCRF-CEM (A) and Ramos cells (B) at pH 6.5 and 7.3 after treatment with 200 nM sgc8, isgc8-5, and Lib in RPMI-1640 media containing 10% FBS for 30 min at 37 °C. (C) Confocal laser scanning microscopy (CLSM) images of CCRFCEM cells treated with 200 nM isgc8-5 in RPMI-1640 media containing 10% FBS for 30 min at 37 °C. Scale bars represent 20 μ m.

Figure 6. Cell viability of CCRF-CEM cells treated with various concentrations of Ce6 modified isgc8-5 at pH 6.5 and 7.3 for PDT. *P* values were calculated by Student's test: ns, nonsignificance, $P > 0.20$, and * for $P < 0.05$, $n = 3$.

Scheme 1.
Schematic Illustration of Structure-Switchable Aptamer for Selective Binding of Receptors on the Surfaces of Target Cells