ATP-mediated Release of a DNA-binding Protein from a Silicon Nanoneedle Array

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1. Introduction

Controlled release is a major goal of drug delivery because release of the drug at the target cells provides a higher active drug concentration and reduces side-effects at non-target cells. Controlled release has been achieved in response to a change in intracellular environment such as pH1 or by external stimulation such as light2 or temperature.3 Environment-responsive materials such as hydrogels,4 aptamer switch for anticancer therapy.12,13 However, there are few reports describing protein delivery. Protein delivery is important both for diagnostics and for genome manipulation with DNA binding molecules such as Zinc-finger proteins (ZFPs).14,15 Here we demonstrate a new method for delivering a ZFP through nanoneedles using a molecular switch based on an ATP aptamer.

2. Experimental

2.1 Zinc-finger protein expression and purification

A ZFP protein fused with the GFP variant mEmerald (ZF-mEm) was expressed in E. coli BL21(DE3) strain carrying the plasmid pET.ZF(C5L)-mEm. pET.ZF(C5L)-mEm was constructed by deleting the Fok I nuclease domain from pET.CCR5.L.FN16 and by inserting the mEmerald fragment from pTagBFP2-mEmerald17 at the Bam HI and Sca I sites. The E. coli cells were cultured at 20°C for 6 h following induction with 0.1 mM IPTG. ZF-mEm proteins were purified by loading the cell extract on a Ni-NTA column equilibrated with 20 mM imidazole and were eluted with 500 mM imidazole elution buffer containing TBSG (10 mM Tris-HCl, 150 mM NaCl, 10 µM ZnCl₂, pH 8.0). The eluted proteins were dialyzed against HBSG (20 mM HEPES, 150 mM NaCl, 10% glycerol, 10 µM ZnCl₂, 10% glycerol, pH 7.4).

2.2 Modification of zinc-finger proteins using a silicon wafer and in vitro release assay

Silicon wafers of 5 mm (for in vitro assay) or silicon nanoneedle arrays of 5 mm square (for in vivo assay) were cleaned with an O₂ plasma asher (J-SCIENCE, JPA300). After rinsing with ultrapure water and EtOH, the nanoneedle array was immersed for 30 min in MPC polymer solution which is comprised of 7% of γ-methacryloyloxypropyl triethoxysilane (MPTES), which binds to silicon, 86% of 2-methacryloyloxyethyl phosphorylcholine (MPC), which inhibits the non-specific absorption of protein,19 and 7% of N-succinimidyl-oxypropyl di(ethylene glycol) methacrylate, which binds to amino
groups in protein. Next, the streptavidin (1 µM) amino groups were reacted with the MPC polymer active esters at r.t. for 1 hr. The molecular switch which is named 5′bio-MS_Fw (10 µM) and two short complementary DNAs (Table 1) were hybridized by incubating at 95°C for 10 min, then decreasing the temperature from 95°C to 25°C at 0.1°C/sec using a thermal cycler and TBS buffer. The 5′biotinylated hybridized molecular switch was supported on a 5 mm² silicon wafer by linkage to streptavidin at r.t. for 60 min. After rinsed with TBS, molecular switch supported nanoneedle array was incubated with ZF-mEm solution at r.t. for 30 min. Intracellular simulation buffer (ISB; 20 mM HEPES, 115 mM CH₃COOK, 5 mM MgCl₂, 1 mM DTT at pH 7.1) was used to assess the release of ZF-mEm by dropping 20 µl of ISB on the ZF-mEm-supported silicon wafer. 2 µl of protein samples were recovered at specified time points and their fluorescence was measured using a fluorospectrometer (Thermo, ND-3300). ISB containing 5 mM ATP and either 20% PEG 200 or PEG 8000 was used for the simulated molecular crowding assays.

2.3 In vivo release assay

HeLa cells expressing red fluorescent protein, DsRed2-NES, whose plasmid was constructed in our laboratory¹⁹ were seeded (10⁵ cells) on a collagen-coated 12Ø glass base dish (IWAKI) and cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS). The medium were changed to DMEM/F-12, which does not contain either phenol red or FBS, prior to insertion of the nanoneedles into the cell. Cells were pierced with the ZF-mEm-supported nanoneedle array, scanned from the tip of nanoneedles upwards 25 µm at a scan rate of 1 µm/slice (total 31 slices) using a confocal laser scanning microscope (CLSM) (IX71/FV300, Olympus). Then, the fluorescence intensity of XY image of the nanoneedles from the tip to 5 µm upper part was analyzed using image analysis software ImageJ. The number of nanoneedles analyzed in this experiment was nine at each condition, either inserted or not inserted.

3. Results and Discussion

3.1 In vitro release assay

ZFP was released from the surfaces of silicon-fabricated nanoneedles in response to ATP using a DNA-based molecular switch comprising a 12 bp ZFP-binding sequence sandwiched between two ATP aptamer sequences. This switch was hybridized with two short complementary oligo DNAs to bind ZFP. When ATPs bind to ATP aptamers, ZFP is released from the molecular switch via conformational change of the ATP aptamers (Scheme 1). ZFP fused with the GFP variant mEmerald (ZF-mEm) was used to monitor ZFP associated with the molecular switch.

To determine whether ZF-mEm can bind to and be released from the molecular switch, we assessed the release of ZF-mEm from the silicon wafer in vitro. The fluorescence intensity of ZF-mEm was measured after addition of HEPES-based buffer containing ATP. We observed a time-dependent and ATP-dose-dependent increase in the relative fluorescence intensity of ZF-mEm (Fig. 1a), whereas no increase in fluorescence intensity was observed when cytidine triphosphate (CTP) was added (Fig. 1b). Therefore, ZF-mEm was selectively released from the silicon wafer in response to ATP. 30

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**Table 1.** Sequence of each component of the molecular switch. The bases shown in bold are bound to ZFP.

<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>5′bio-MS_Fw</td>
<td>ACCTGGGGGAGTATTGCGGAGGAAGGTGATGAGGATGACACCTGGGGGAGTATTGCGGAGGAAGGT</td>
</tr>
<tr>
<td>Comp.R1</td>
<td>CTCACTACCTTCTTCT</td>
</tr>
<tr>
<td>Comp.R2</td>
<td>CCCAGGTGTCACT</td>
</tr>
</tbody>
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**Figure 1.** (Color online) In vitro ZFP release from a silicon wafer. (a) ATP dose- and time-dependent increase in fluorescence intensity. (b) Fluorescence intensity change following the addition of 5 mM ATP or CTP. After 30 min, the volume of ISB remaining on the silicon wafer was replaced with 10 mM ATP or CTP. Relative fluorescence intensity means the ratio of fluorescence intensity when the fluorescence intensity after 60 min in (a) and 90 min in (b) is set to 1.
Figure 2. (Color online) ZFP release from the silicon wafer under molecular crowding conditions in vitro. Relative fluorescence intensity means the ratio of the fluorescence intensity when the maximum fluorescence intensity in the absence of PEG condition is set to 1.

Figure 3. (Color online) ZFP release from a nanoneedle array in vivo. (a) CLSM images of ZF-mEm modified nanoneedles 0 min and 30 min after insertion into HeLa cells expressing DsRed. Scale bars are 5 µm. (b) Time course of the relative fluorescence intensities of nanoneedles following insertion into the cell (n = 9). Background fluorescence intensity is subtracted in each plot. Initial fluorescence of the nanoneedle is assigned a value of “1”. \*P = 0.01.

minutes incubation was sufficient for binding of ATP (2.5–10 mM) to the aptamer moiety as reported in the previous studies.\(^\text{13,20,21}\) Since the concentration of ATP in a cell is assumed to be 1–10 mM,\(^\text{9,10}\) we had expected that in vivo ZFP release could be achieved by binding of intracellular ATP.

3.2 In vitro release assay in a simulated intracellular environment

Next, we assessed whether ZF-mEm can be released from the silicon wafer under conditions approximating the intracellular environment by using ISB, which contains polyethylene glycol (PEG) as a macromolecular crowding reagent.\(^\text{22}\) Little release of ZF-mEm was observed in ISB containing 5 mM ATP and 20% PEG compared with buffer without PEG (Fig. 2), indicating that the binding affinity between DNA and the binding protein increases in a macromolecular crowding environment, as reported previously.\(^\text{23}\)

3.3 In vivo release assay using human cultured cells

The release of ZF-mEm from a nanoneedle array inserted into cells was observed using HeLa cells expressing DsRed by measuring the fluorescence of ZF-mEm on the nanoneedle by CLSM at timed intervals. The fluorescence intensity of inserted nanoneedles decreased more rapidly than that of non-inserted nanoneedles (Fig. 3). The extracellular concentration of ATP is less than 5 µM.\(^\text{11}\) Thus, release of ZF-mEm caused by ATP in medium is negligible. Decreasing of fluorescence intensity of non-inserted nanoneedles might be attributed to photobleaching of Emerald caused by the laser irradiation of CLSM.\(^\text{24}\) The net release of ZF-mEm after 30 min was approximately 20%, which corresponds to at most 10\(^4\) molecules into a single cell based on our calibration curve for the fluorescence intensity of ZF-mEm, although there was significant scatter in the data. This scatter may be due to differences in ATP concentration in the intracellular organelles or due to the cell cycle phases of each cell.\(^\text{32}\) Moreover, the slower release of ZF-mEm in the in vivo assay is likely caused by macromolecular crowding.

4. Conclusions

We here demonstrated a new method for releasing ZFP from silicon in response to ATP by delivering ZFP into cells using nanoneedles containing surface-immobilized DNA-based ATP aptamers. Approximately 20% of the net ZF-mEm was released. Intracellular molecular crowding apparently affects the interaction between DNA and the binding protein. Our nanoneedle approach should expand the ability to deliver biomolecules into cells and will require devising silicon surfaces with specific molecular switches that respond to the intracellular environment.

References