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Programmable Release of Multiple Protein Drugs from Aptamer-Functionalized Hydrogels via Nucleic Acid Hybridization

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Supporting Information

ABSTRACT: Polymeric delivery systems have been extensively studied to achieve localized and controlled release of protein drugs. However, it is still challenging to control the release of multiple protein drugs in distinct stages according to the progress of disease or treatment. This study successfully demonstrates that multiple protein drugs can be released from aptamer-functionalized hydrogels with adjustable release rates at predetermined time points using complementary sequences (CSs) as biomolecular triggers. Because both aptamer−protein interactions and aptamer−CS hybridization are sequence-specific, aptamer-functionalized hydrogels constitute a promising polymeric delivery system for the programmable release of multiple protein drugs to treat complex human diseases.

Protein drugs can cause severe side effects in normal tissues and organs, despite their great potential for the treatment of various human diseases.[1] Therefore, great efforts have been made to develop polymeric delivery systems to achieve localized and controlled release of protein drugs.[2] Current polymeric systems release protein drugs via one of two mechanisms: preset control or real-time control. Preset control depends mostly on the pore size and/or degradation rate of a polymeric delivery system, which is predetermined during its synthesis.[3] This approach is suitable for applications needing sustained drug release. However, it is challenging to apply this mechanism to adjust the protein release time and rate according to the progress of disease or treatment. This difficulty can be overcome by using an external stimulus for real-time control. The stimulus can be temperature variations, electric potentials, magnetic fields, ultrasound, or irradiation.[4] In response to stimulation, polymeric systems change their structural integrity, volume, or pore size and release protein drugs accordingly. While these mechanisms have been successfully applied to achieve precise control of the release of single proteins, they lack specificity in regulating the release of multiple protein drugs in distinct stages, which is often necessary to treat complex human diseases.[5] Therefore, it is important to develop novel polymeric delivery systems that allow for the controlled release of multiple protein drugs at desired time points.

The purpose of this study was to demonstrate that aptamer-functionalized hydrogels can be programmed to release multiple proteins when needed through nucleic acid hybridization. Nucleic acid aptamers are single-stranded oligonucleotides that are screened from DNA/RNA libraries to bind to various target molecules.[6] Aptamers have received tremendous attention in various fields[7] because they have high affinities and specificities comparable to those of antibodies.[8] More importantly, unlike other affinity ligands, nucleic acid aptamers can hybridize with complementary sequences (CSs). As a result, rationally designed CSs can regulate the binding functionality of aptamers and induce the rapid dissociation of aptamer−protein complexes.[9] Therefore, it was hypothesized that multiple aptamers could entrap different protein drugs because of their high binding affinities and specificities and that CSs could function as sequence-specific molecular triggers to program precisely the release of multiple protein drugs at desired time points (Figure 1).

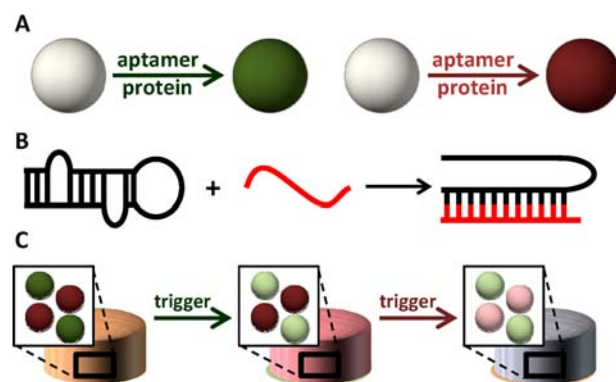

Figure 1. Schematic of the concept. (A) Preparation of aptamer-functionalized particles for protein loading. (B) Intermolecular aptamer−CS hybridization. (C) Programmable release of multiple proteins from aptamer-functionalized hydrogels at desired time points. The color intensity of the balls indicates the amount of proteins. The light colors indicate fewer proteins.

To test the hypothesis, two aptamer models were used in this study. These two aptamers have been well-studied and bind to vascular endothelial growth factor (VEGF) and platelet-derived growth factor BB (PDGF-BB).[10] They were used to functionalize particles and bind VEGF and PDGF-BB, respectively. The particles were physically incorporated into a hydrogel network to form aptamer-functionalized hydrogels. In this proof-of-concept study, streptavidin-coated polystyrene microparticles

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were used as a model. For real applications, they would need to be substituted with biocompatible, biodegradable, and porous microparticles to improve the biocompatibility and increase the protein loading efficiency.

The hydrogels were subjected to a protein release test in the absence of trigger molecules to examine the function of the nucleic acid aptamers in binding to and holding the protein drugs within the hydrogel. As shown in Figure 2A, more than 50% of the VEGF was released from the native hydrogel and the anti-PDGF-BB aptamer-functionalized hydrogel during the first day. In the following four days, an additional 20% was released before the release profiles approached a plateau. In contrast, ~25% of the VEGF was released from the anti-VEGF aptamer-functionalized hydrogel during the first day and 8% between days 2 and 5. These results clearly show that the anti-VEGF aptamer significantly reduced the initial burst release and efficiently entrapped VEGF in the hydrogel. In addition, the small difference between the native hydrogel and the anti-PDGF-BB aptamer-functionalized hydrogel in controlling the release of VEGF demonstrates the binding specificity of the aptamers. Similarly, the anti-PDGF-BB aptamer efficiently entrapped PDGF-BB within the hydrogel. During the first day, the amount of PDGF-BB released from the native hydrogel was 55%, ~4 times as much as that released from the anti-PDGF-BB aptamer-functionalized hydrogel. In the following 4 days, the PDGF-BB release from the native hydrogel was 10%, whereas the release from the anti-PDGF-BB aptamer-functionalized hydrogel was less than 3%. It was also observed that PDGF-BB release from the anti-VEGF aptamer-functionalized hydrogel was slower than that from the native hydrogel. The isoelectric point of PDGF-BB is 9.5, which leads to a net positive charge under neutral conditions.[11] Thus, it is possible that electrostatic interactions between PDGF-BB and the anti-VEGF aptamer led to the slower PDGF-BB release in comparison with that from the native hydrogel. However, PDGF-BB release from the anti-VEGF aptamer-functionalized hydrogel was much faster than that from the anti-PDGF-BB aptamer-functionalized hydrogel. Taken together, these results demonstrate that nucleic acid aptamers can efficiently entrap protein drugs in hydrogels and slow their release.

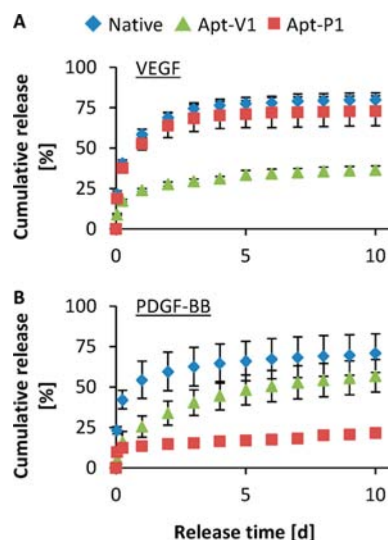

Figure 2. Protein release from hydrogels. (A) VEGF release. (B) PDGF-BB release.

After demonstrating that aptamers have the capability to retain protein drugs in hydrogels, we studied whether or not CSs would bind specifically to the corresponding aptamers in the hydrogel. To answer this question, the particles and the hydrogels were incubated with fluorophore-labeled CSs. The hybridizing functionalities of the CSs were characterized using flow cytometry and fluorescence microscopy. The flow cytometry results show that the particles interacted with the corresponding CSs rather than noncomplementary sequences and exhibited large fluorescence shifts in the flow cytometry histograms (Figure S1A,B in the Supporting Information). Consistent with the flow cytometry analysis, the fluorescence images show that the aptamer-functionalized hydrogels exhibited the fluorescence signal of the CSs (Figure S1C). Therefore, it is clear that CSs can penetrate hydrogels and hybridize with aptamers with high specificity.

A series of CSs (Table S1) were designed to investigate the efficacy of CSs in disassembling aptamer−protein complexes to trigger protein release from hydrogels. Each of three CSs that hybridize with ~40% of the anti-VEGF aptamer was applied to

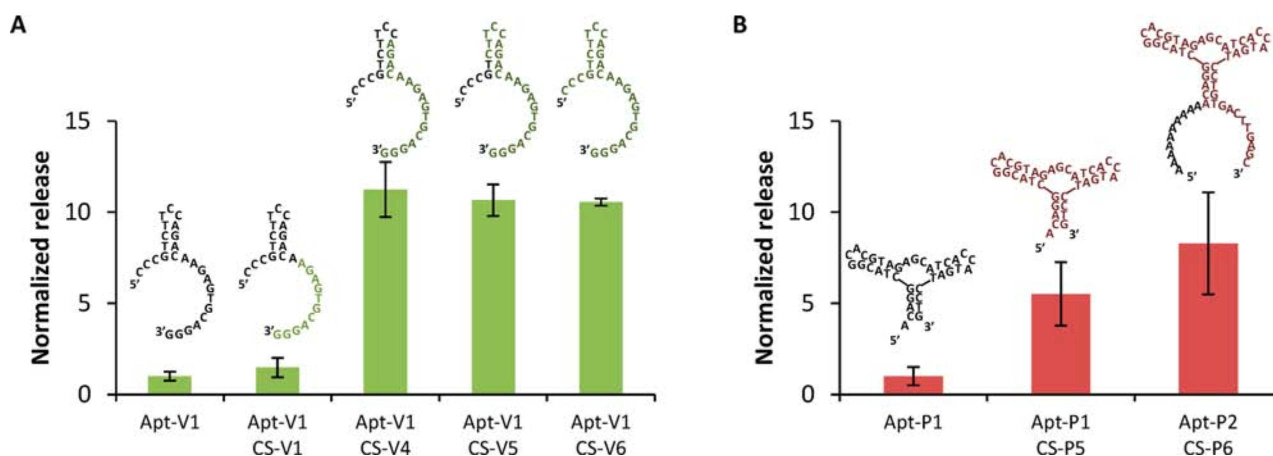

Figure 3. Release control via nucleic acid hybridization. (A) VEGF release triggered by CSs with different lengths. (B) Effect of increasing hybridization length on triggered PDGF-BB release. The release of PDGF-BB could be further increased when a tail was added to the anti-PDGF-BB aptamer and the hybridization length was increased. The colored region on the aptamer structure shows the nucleotides of the aptamer that hybridize with the CS. The triggered release was normalized against the nontriggered release (i.e., no CS applied).

trigger VEGF release. Although the electrophoretogram shows that these CSs could hybridize with the aptamer, none of these sequences was able to trigger VEGF release (Figure S2). Likewise, the short, oligonucleotides complementary to the anti-PDGF-BB aptamer could not trigger PDGF-BB release (Figure S2). The difference between the gel electrophoresis results and the release results lies in the presence of target proteins in the release system, where intermolecular nucleic acid hybridization competes against the aptamer−protein interaction. When the latter is stronger than the former, it is too difficult to trigger aptamers to release the bound protein drugs. Therefore, the results suggest that it is difficult for short CSs to trigger the dissociation of aptamer−protein complexes because of the strong interactions between the aptamers and the proteins.

The number of base pairs directly affects the strength of intermolecular hybridization.[12] Thus, the length of the CSs was increased to strengthen their competitive capability. When the length of the CS was increased from 11 to 16 nucleotides, the rate of VEGF release was increased by 1 order of magnitude (Figure 3A). Any further increase in the length of the CS did not produce a faster release. The release rate of PDGF-BB also increased with increasing CS length (Figure S3). When the CS length was increased to that for complete hybridization of the anti-PDGF-BB aptamer, the release rate of PDGF-BB was increased to nearly 6 times that for protein release from the nontriggered hydrogel. These results suggest that increasing the length of nucleic acid hybridization is effective in inducing aptamer−protein dissociation for triggered protein release. However, the magnitude of triggered PDGF-BB release was less than that of VEGF.

To enhance the triggered PDGF-BB release, the length of the anti-PDGF-BB aptamer and the hybridization length were increased. An additional 10 nucleotides were attached to both the 3′ and 5′ ends of the anti-PDGF-BB aptamer. The nucleotides added to the 3′ end were used to increase the hybridization length. The adenosines added to the 5′ end were used as a spacer to reduce the likelihood of steric hindrance during intermolecular hybridization on the particle surface. The structural prediction showed that the addition of these extra nucleotides would not affect the original stem−loop structure of the 36-nucleotide aptamer (Figure 3B), indicating that the binding functionality of the aptamer would not be affected by the added nucleotides. The release results show that the addition of nucleotides to the aptamer and the use of a longer CS led to a higher rate of PDGF-BB release (Figure 3B). In contrast, when extra nucleotides were added to the anti-VEGF aptamer, triggered VEGF release was not enhanced with the increased hybridizing length (Figure S4). These results indicate that the design of an effective CS to trigger protein release is aptamer-specific.

The difference in the CS designs needed to trigger these two aptamers effectively may be attributed to their binding affinities and functional structures. The initial release test showed that less PDGF-BB than VEGF was released during the same period of time (Figure 2), indicating that the anti-PDGF-BB aptamer has a higher binding affinity than the anti-VEGF aptamer. Thus, it is reasonable that a longer CS would be needed to compete against and trigger the release of PDGF-BB. In addition, the anti-PDGF-BB aptamer exhibits a secondary structure that has three stems with a total of 13 base pairs, whereas the anti-VEGF aptamer has only one stem comprising four base pairs. The higher numbers of stems and base pairs lead to stronger intramolecular hybridization. As a result, it is difficult for short CSs to hybridize with aptamers bearing a strong degree of intramolecular hybridization. When nucleotides that do not form intramolecular base pairs are added to an aptamer, they can function as a linear anchoring site to promote the binding of the CS to the aptamer. The anti-VEGF aptamer has a high percentage of unpaired nucleotides, hanging at both the 3′ and 5′ ends. These nucleotides may play the role of the anchoring site, which is the likely explanation of why the addition of extra nucleotides to this aptamer did not enhance the triggered VEGF release. In contrast, the 3′ and 5′ ends of the anti-PDGF-BB aptamer form a stem and do not possess a single-stranded tail. Therefore, the addition of nucleotides to the anti-PDGF-BB aptamer facilitated nucleic acid hybridization and triggered release.

In addition to the rational design of the aptamer and CS sequences, triggered release can be modulated by other parameters such as triggering time and the CS concentration. For instance, when the triggering time is prolonged, the amount of released protein would be expected to increase, which would in turn require a lower CS concentration to trigger the same amount of protein release. Because CSs play an important role in this concept, it is reasonable to raise a concern about the feasibility of using CSs in vivo. Oligonucleotides have little immunogenicity and toxicity because they are small in size and composed of nucleotides. Clinical trials have shown that the human body can tolerate micromolar levels of oligonucleotides.[13] Thus, the use of CSs is expected to be feasible for in vivo applications. All of these issues will be systematically studied in future works.

After the examination of CS-mediated triggering efficacy, the two aptamer-functionalized particles were incorporated into the hydrogel to study programmed protein release. The confocal micrograph in Figure 4A shows that these two particles were randomly distributed in the hydrogels without obvious particle aggregation. The hydrogels were treated for 1 h at two different time points, first with CS-V6 and then with CS-P6. The results show that the daily release rate of VEGF after triggering with CS-V6 on day 4 increased from 1% to ∼14%, whereas the

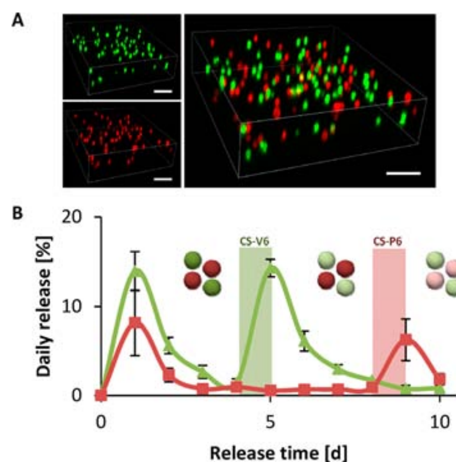

Figure 4. Programmable release of VEGF and PDGF-BB. (A) Laser confocal micrograph of the two aptamer-functionalized particles in the hydrogel. Scale bars: 10 μm. (B) Profiles of daily release of VEGF (green) and PDGF-BB (red) regulated via sequence-specific CSs. CS-V6 and CS-P6 were added to the release medium on days 4 and 8, respectively. Each triggering time was 1 h.

PDGF-BB release was not affected (Figure 4B and Figure S5). Similarly, the daily release rate of PDGF-BB after triggering with CS-P6 on day 8 increased from 0.5% to ~6%, whereas the VEGF release was not affected (Figure 4B and Figure S5). These data clearly show that VEGF and PDGF-BB were released in a programmable manner using sequence-specific nucleic acid hybridization.

Various hormones and growth factors are produced by the body at distinct stages rather than constantly.[14] A typical example is angiogenesis, which requires the presence of multiple growth factors at different stages.[5] At the beginning of angiogenesis, certain growth factors (e.g., VEGF) are needed to promote the proliferation and migration of endothelial cells to form new vessels. At a later stage, other types of growth factors (e.g., PDGF-BB) are needed to stabilize the newly formed vessels. Therefore, the ability to modulate the sequential release of VEGF and PDGF-BB from aptamer-functionalized hydrogels may have a direct impact on the development of clinical protocols for therapeutic angiogenesis.

In summary, we have successfully demonstrated that two different nucleic acid aptamers and CSs can be applied to control the release of two types of protein drugs in predetermined stages from the same hydrogel. Although two aptamer models were used in this study, more than two aptamers and protein drugs could in principle be incorporated into the hydrogels. Because both aptamer−protein interactions and aptamer−CS hybridization are sequence-specific, aptamer-functionalized hydrogels are a promising platform for the controlled release of multiple protein drugs with adjustable release rates at desired time points, which could benefit the treatment of various complex human diseases.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, table of oligonucleotide sequences, and additional experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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