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Cell adhesion on an artificial extracellular matrix using aptamer-functionalized PEG hydrogels

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ABSTRACT

The development of an artificial extracellular matrix (ECM) is important to regenerative medicine because the ECM plays complex and dynamic roles in the regulation of cell behavior. In this study, nucleic acid aptamers were applied to functionalize hydrogels for mimicking the adhesion sites of the ECM. The results showed that nucleic acid aptamers could be incorporated into polyethylene glycol (PEG) hydrogels via free radical polymerization. The incorporation of the aptamers produced only a moderate effect on the mechanical properties of the PEG hydrogels. Importantly, the results also showed that the aptamers effectively induced cell type-specific adhesion to the PEG hydrogels without affecting cell viability. The cell adhesion was a function of the aptamer concentration, the spacer length and the cell seeding time. In addition, cell adhesion to the aptamer-functionalized hydrogel could be attenuated by means of aptamer inactivation in a physiological condition. Thus, aptamer-functionalized hydrogels are promising biomaterials for the development of artificial ECMs.

1. Introduction

The integration of bioactive molecules and/or living cells into synthetic materials has led to the development of a variety of biomimetic and bioinspired materials [1–3]. The study of artificial ECMs has particularly attracted significant attention because of their potential applications in the field of regenerative medicine [3–5]. Natural ECMs are comprised of insoluble macromolecules (*e.g.,* proteoglycans), soluble signaling molecules (*e.g.,* growth factors), and adhesion ligands (*e.g.,* fibronectin) [6]. These ECM components play complex and dynamic roles in tissues. Based on the communication with these components, cells acquire essential biochemical and biophysical cues that determine the behavior of the cells [6]. Hydrogels have been widely studied as a fundamental support component for the synthesis of artificial ECMs [7,8], because they have structural similarities to human tissues with tunable viscoelasticity and high permeability for molecular transport. However, most hydrogels do not possess the essential biochemical and/or biophysical cues required for regulating cell behavior. It is therefore necessary to incorporate biofunctional molecules into the hydrogel network to simulate the natural regulatory processes of ECMs.

The dissolution of signaling molecules (*e.g.,* growth factors) into a hydrogel is a common way to incorporate biochemical cues into an artificial extracellular matrix [9]. However, most hydrogels are highly permeable in nature. Though this characteristic is advantageous for the molecular transport of nutrients and wastes to support cell survival and intercellular communication, it is disadvantageous for the long-term release of signaling molecules to provide cells with sufficient biochemical stimuli. To solve this problem, polymeric microparticles that can control the release of growth factors have been used to functionalize hydrogels [10]. Alternatively, hydrogels can be modified with heparin, Ni^{2+}, or peptides that can physically absorb positively charged or histidine-tagged signaling molecules to achieve the sustained release [11–13]. Hydrogels can also be functionalized with nucleic acid aptamers to control the release of growth factors [14–17]. Nucleic acid aptamers are single-stranded oligonucleotides selected from the libraries of synthetic oligonucleotides [18,19]. They have numerous merits such as high affinity, high specificity, and little immunogenicity. In addition, they can be chemically modified to acquire resistance against nuclease degradation [20]. Studies also show that the release of growth factors from aptamer-functionalized hydrogels can be triggered by PEGylated complementary oligonucleotides at predetermined multiple time points [16]. Therefore, it is promising that multiple

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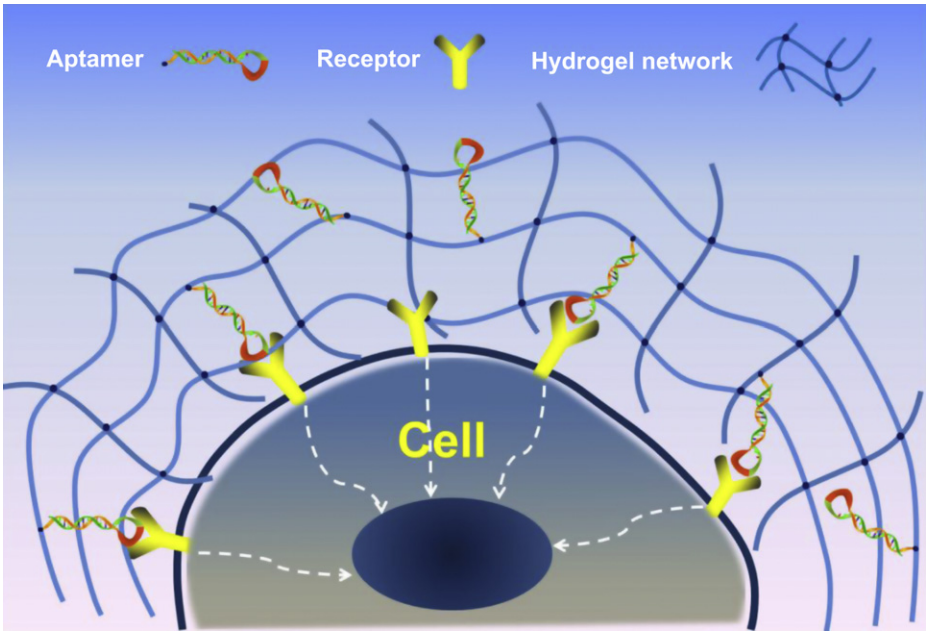

Fig. 1. Schematic of cell adhesion to aptamer-functionalized hydrogel.

growth factors can be incorporated into aptamer-functionalized hydrogels as biochemical cues to mimic the sustained and/or triggered release functionality of ECMs.

In addition to the necessity of incorporating soluble signaling molecules into the hydrogels, it is also important to functionalize hydrogels with adhesion ligands to provide cells with biophysical cues. Natural adhesion proteins or small adhesion peptide sequences identified within the adhesion proteins have been used to modify hydrogels for this purpose [3]. Though natural adhesion proteins can provide cells with an environment similar to the native extracellular microenvironment, they are often limited by fast degradation [21]. In addition, the proteins may lose the capability of recognizing their cognate receptors during the material synthesis. For instance, collagen is one of the critical components in the ECM, interacting with numerous cell surface receptors [22]. However, recent experimental results have indicated that collagen sponges have to be further functionalized with adhesion peptides to improve their cell recognition function [23]. Small adhesion peptides have merits such as easy synthesis, easy conjugation, and little immunogenicity, but they often require circularization, multimerization, and presentation in the context of "parent" proteins in order to achieve sufficient structural stability and binding affinity [21]. Therefore, it is desirable to explore new biomolecular ligands to functionalize hydrogels and mimic the biophysical cues of ECMs.

The long-term goal of this study is to develop an artificial ECM using hydrogels and nucleic acid aptamers. The aptamers are expected to not only control the release of multiple growth factors (*i.e.*, biochemical cues), but also provide cells with cell binding sites (*i.e.*, biophysical cues). Because our previous research demonstrated that aptamer-functionalized hydrogels could allow for the temporal control of growth factors with desired release kinetics [14–17], this study was focused on studying the feasibility of using aptamers as the binding sites of hydrogels for cell adhesion (Fig. 1). PEG hydrogels were used as the model material system due to their biocompatibility and their wide applications in the field of biomaterials [24]. Aptamers were incorporated into the PEG hydrogels through free radical polymerization. The PEG hydrogels were characterized by rheology, fluorescence microscopy, and a swelling test. The effects of various parameters on cell adhesion to the aptamer-functionalized PEG hydrogels were also studied.

2. Materials and methods

2.1. Chemical reagents

Poly(ethylene glycol) diacrylate (PEGDA; average M_n: 700 Da) and 3-(trimethoxysilyl)propyl methacrylate (TMSPM) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS), ammonium persulfate (APS), N,N,N′,N′-tetramethylenediamine (TEMED), and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Suwanee, GA). All oligonucleotides (Table 1) were purchased from Integrated DNA Technologies (Coralville, IA).

2.2. Prediction of secondary structures

The secondary structures of the aptamers were generated by using RNAstructure version 5.0 (http://rna.urmc.rochester.edu/rnastructure.html). The predicted structures with the lowest free energies were presented.

2.3. Synthesis of PEG hydrogels

PEG hydrogels were synthesized on a glass surface for the convenience of operating cell adhesion experiments. Glass slides were cut to the dimension of approximately 8 mm × 8 mm. The glass was cleaned using acetone and 1 M NaOH solution sequentially. Acrylate groups were generated on the glass surface through silanization as previously described [25]. In brief, the silanization solution was prepared by mixing 0.5 mL TMSPM in 50 mL ethanol with 1.5 mL of 10% diluted glacial acetic acid. After the

Table 1
Sequences of oligonucleotides.

Name	Sequence (5′→3′)
sgc8c-10A	/*acrydite*/AAAAAAAAAATCTAACTGCTGCGCCGCCG GGAAAATACTGTACGGTTAGA
sgc8c-5A	/*acrydite*/-AAAAATCTAACTGCTGCGCCGCCGGGAA AATACTGTACGGTTAGA
sgc8c-0A	/*acrydite*/-TCTAACTGCTGCGCCGCCGGGAAAATAC TGTACGGTTAGA
ACA	/*acrydite*/-GCGATACTCCACAGGCTACGGCACGTAG AGCATCACCATGATCCTG
FAW	CACTTAGAGTTCTAACTGCTGCGCCGCCGGGAAAAT ACTGTACGGTTAGA
CO-FAM	/*FAM*/-TCTAACCGTACAGTATTTTC
sgc8c-FAM	/*FAM*/-TCTAACTGCTGCGCCGCCGGGAAAATACTGTA CGGTTAGA
CO	TCTAACCGTACAGTATTTTC

The binding region of the aptamers is underlined. ACA: acrydited control aptamer; FAW: functional aptamer without acrydite.

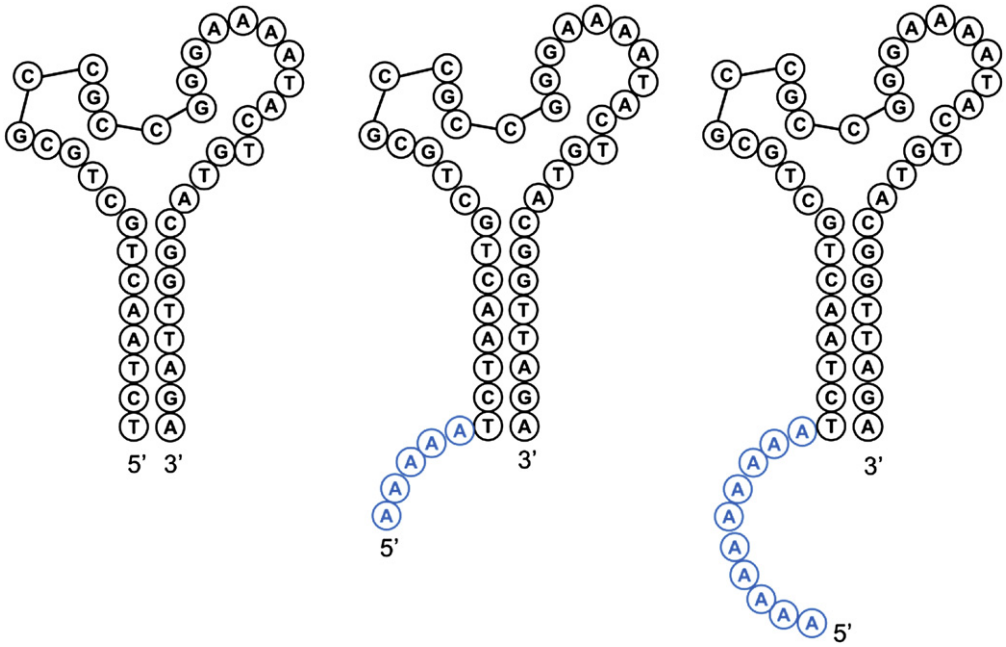

Fig. 2. Secondary structures of aptamers. Left: sgc8c-0A; middle: sgc8c-5A; right: sgc8c-10A. The structures were generated by using RNAstructure version 5.0. The structures with the lowest free energies are presented. The tail of the aptamer is marked in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

glass slides were incubated in the silanization solution for 5 min, the slides were thoroughly rinsed with ethanol, dried in air, and stored in a desiccator before use.

PEGDA was diluted in PBS to a final concentration of 20% w/v. The reaction solution was 5 μL of the PEGDA and aptamer mixture, 0.15 μL APS (10% w/v), and 0.15 μL TEMED (50% v/v). Immediately after the preparation of the reactive solution, the solution was deposited on a large glass slide and subsequently covered by the silanized glass slide. The PEG hydrogel tightly attached to the silanized glass slide because the silanized glass surface had acrylate groups that were incorporated into the polymer network. After curing for 2 h, the silanized glass slide was lifted from the larger glass slide. Hydrogel without any modification, hydrogel functionalized with control aptamers, and hydrogel encapsulating aptamers without the acrydite group were prepared in the same way as control hydrogels.

2.4. Gel electrophoresis

The solutions of aptamers and complementary oligonucleotides (COs) were pretreated by one cycle of heating (95 °C for 1 min) and cooling (room temperature for 1 h). Aptamers were then mixed with COs at a molar ratio of 1:1 and incubated at 37 °C for 1 h. The mixture was loaded into a 10% native polyacrylamide gel for gel electrophoresis in a Bio-Rad Mini-PROTEAN tetra cell (Hercules, CA). After electrophoresis, the polyacrylamide gel was stained with ethidium bromide and then imaged with a Bio-Rad GelDoc XR system (Hercules, CA).

2.5. Fluorescence imaging of hydrogels

Complementary oligonucleotides labeled with 6-carboxy-fluorescein (denoted as CO-FAM) were used as a probe to hybridize with the aptamers in the hydrogels to examine the incorporation of the aptamers into the PEG hydrogel. The hydrogel samples were treated by gel electrophoresis and also thoroughly washed to remove any unreacted aptamers. The hydrogels were then treated with CO-FAM solution for 1 h. After thorough washing, the samples were imaged using the CRI Maestro EX SYSTEM (Woburn, MA). The images were analysed with the software provided by the manufacturer.

2.6. Rheology

The samples used in the rheology analysis were prepared as circular discs of 20 mm in diameter and 0.75 mm in thickness. The procedure of hydrogel preparation was the same as described above. The aptamer concentration in the hydrogel was 2 μM. The storage (G′) and loss (G″) moduli of the hydrogels were measured with an AR-G2 rheometer (TA Instruments, New Castle, DE). A stress-sweep test was performed to confirm that the measurement was in the linear viscoelastic regime. The oscillation stress was varied from 0.01 to 1000 Pa at a fixed frequency of 1 rad/s and the frequency was varied from 0.5 to 100 rad/s at an oscillation stress of 1 Pa oscillation stress. The temperature was set at 37 °C for all experiments.

2.7. Swelling examination

Hydrogels of different aptamer concentrations were prepared in a cylindrical mold at room temperature. The hydrogel samples were incubated in PBS (pH 7.4) at 37 °C after dried in vacuum to a constant weight (W_i). At predetermined time points, the samples were taken out of the PBS solution and blotted with tissue paper to remove excess water for the measurement of wet weights (W_s). The swelling was determined by using the following equation:

$$\text{Swelling ratio } (\%) = \left[\frac{W_s - W_i}{W_i}\right] \times 100$$

2.8. Cell culture

CCRF-CEM (CCL-119; human T lymphoblast cell line) and Ramos cells (CRL-1596; human B lymphocyte cell line) were obtained from ATCC (Manassas, VA). Both cell lines were cultured and maintained in RPMI medium 1640 (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum and 100 units/mL of penicillin–streptomycin (Mediatech, Manassas, VA) at 37 °C in a 5% CO_2 atmosphere. All reagents used for cell culture were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted.

2.9. Flow cytometry

Flow cytometry was performed using the same procedure as previously described [26]. Briefly, approximately 5×10^5 cells were incubated in the binding solution for 1 h at 4 °C. The binding solution contained 50 nM of FAM labeled aptamers or control aptamers. After washing with 1 mL cold binding buffer, the labeled cells were immediately analysed by flow cytometry (BD FACSCalibur flow cytometer, San Jose, CA). A total of 10, 000 events were counted.

2.10. Examination of cell adhesion

The buffer used for cell adhesion was Dulbecco's phosphate buffered saline (DPBS) supplemented with 4.5 g/L of glucose, 5 mM $MgCl_2$ and 0.1% (w/v) BSA. The hydrogel samples were transferred into a 24-well plate and incubated with a cell suspension (5×10^5 cells/well) at 37 °C. At a predetermined time point, the hydrogel samples were taken out of the wells for washing. The washing was conducted to remove the unbounded or loosely bounded cells by shaking at 90 rpm for 10 min in a New Brunswick shaker (Edison, NJ). Cell images were captured using an inverted microscope (Axiovert 40CFL, Carl Zeiss) and also analysed with the software Image J to examine the number of cells attached to the hydrogel.

Two additional experiments were performed to better understand cell-hydrogel interactions. One experiment was to treat cells with trypsin prior to the cell adhesion test. Approximately 5×10^5 CCRF-CEM cells were incubated in 500 μL of trypsin solution containing trypsin (0.05%) and EDTA (0.53 mM) at 37 °C. After 15 min of

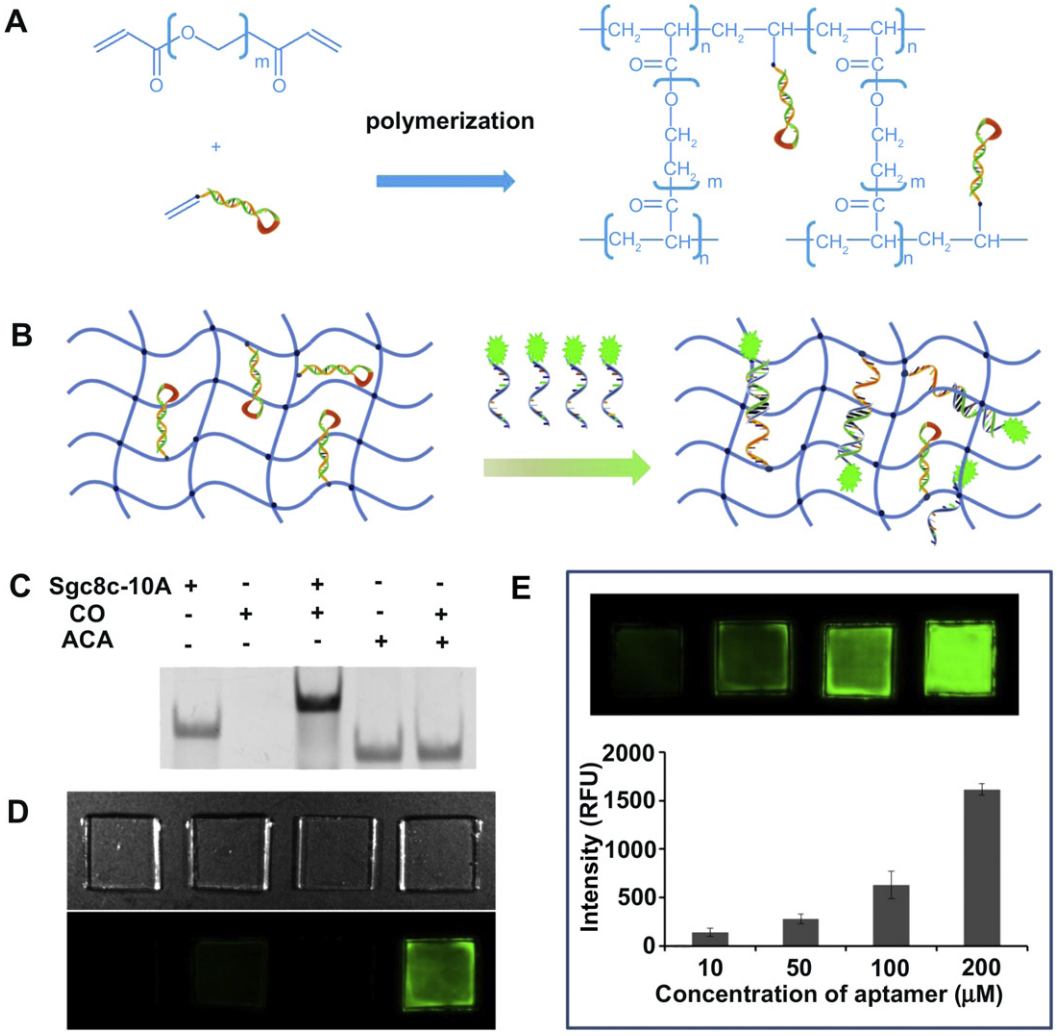

Fig. 3. Synthesis of aptamer-functionalized PEG hydrogel. (A) Schematic of incorporating aptamers into the PEG hydrogel network via free radical polymerization. (B) Schematic of fluorescence labeling of aptamer-functionalized PEG hydrogel. (C) Gel electrophoresis analysis of intermolecular hybridization between the sgc8c-10A aptamer and its complementary oligonucleotide. CO: Complementary oligonucleotide; ACA: acrydited control aptamer. (D) Fluorescence imaging of the PEG hydrogels after gel electrophoresis and hybridization with CO-FAM. Top: monochrome images; bottom: fluorescence images. Type of hydrogel (from left to right): Hydrogel without the aptamers; hydrogel physically encapsulating the aptamers (*i.e.*, FAW); hydrogel functionalized with the ACA; hydrogel functionalized with the functional aptamers (*i.e.*, sgc8c-10A). The molar ratio of CO-FAM to aptamer was 1:1. (E) Fluorescence imaging of PEG hydrogel functionalized with sgc8c-10A of different concentration. The molar ratio of CO-FAM to aptamer was 1:1.

incubation, the trypsin was neutralized by the cell culture medium containing fetal bovine serum and removed with washing. The cells were subsequently used in the cell adhesion test. Cells treated with the binding buffer were used as a control. Another experiment was carried out based on the pre-treatment of the aptamer-functionalized hydrogels with the COs. Hydrogel samples were incubated in a CO solution at 37 °C for 1 h. After thorough washing to remove unhybridized COs, the cells were seeded onto the PEG hydrogels. The procedures of capturing cell images and examining cell adhesion were the same as described above.

2.11. LIVE/DEAD cell staining

Cells were seeded on the PEG hydrogels functionalized with 50 μM aptamer for 30 min. At a predetermined time point, the cells were stained with a mixture of calcein AM (1 μM) and ethidium homodimer-1 (1 μM) using the kit of LIVE/DEAD cell assay purchased from Invitrogen (Carlsbad, CA). Fluorescent cell images were captured using an inverted microscope (Axiovert 40CFL, Carl Zeiss).

3. Results

3.1. Prediction of secondary structures

The aptamer used in this study is a truncated version (denoted as sgc8c) of the full-length aptamer that was originally selected from a DNA library [27]. This aptamer was purposely chosen because its binding functionality is well characterized. To understand the steric effect on aptamer–cell interaction, the sgc8c aptamer was also modified with the addition of a chain of adenosines (A) to its 5′ end. The predicted secondary structures are shown in Fig. 2. The sgc8c aptamer has a long stem, a 12-nucleotide loop, and a small bulge located between the stem and the loop. The 5A and 10A tail attached to the sgc8c aptamer were predicted not to participate in any intramolecular hybridization, suggesting that the two sgc8c aptamer derivatives exhibit the same binding functionality as the sgc8c aptamer.

3.2. Synthesis of aptamer-functionalized PEG hydrogels

The 5′ ends of the aptamers were initially functionalized with an acrydite group for their chemical incorporation to the PEG hydrogels. The double bond of the acrydite group is activated during free radical polymerization and the activated double bonds of the aptamers and PEGDAs are linked together to form the PEG hydrogel (Fig. 3A). An intermolecular hybridization assay was used to

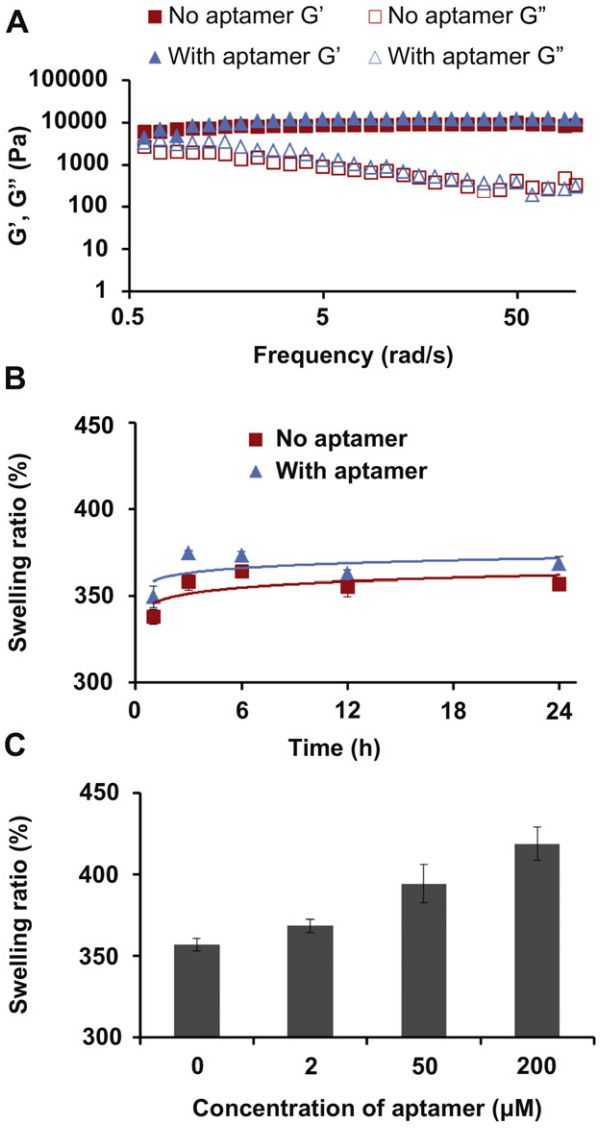

Fig. 4. Characterization of mechanical properties. (A) Rheology analysis of moduli. G′: storage modulus; G″: loss modulus. The frequency was varied from 0.5 to 100 rad/s at an oscillation stress of 1 Pa oscillation stress. The aptamer concentration was 2 μM. (B) Dynamic swelling of hydrogels. The aptamer concentration was 2 μM. (C) Effect of aptamer concentration on swelling. The hydrogel samples were examined 24 h after incubated in PBS solution. Four samples were studied in each group.

characterize the hydrogel synthesis and to demonstrate the success of incorporating the acrydited aptamers into the PEG hydrogels (Fig. 3B). In this assay, the hydrogel was treated with CO-FAM for 1 h followed by thorough washing. As shown in Fig. 3C, the COs and the sgc8c-10A aptamers could efficiently hybridize in an aqueous solution. Thus, if the aptamers were successfully incorporated into the PEG hydrogels, CO-FAM would be able to hybridize with the incorporated aptamers and stain the PEG hydrogels with green fluorescence. Otherwise, the free aptamers would be easily removed from the PEG hydrogels during the washing procedure and the hydrogels would not catch the CO-FAM to emit a strong fluorescence signal. The fluorescence images showed that the aptamer-functionalized PEG hydrogel had stronger fluorescence than the control hydrogels (Fig. 3D). In addition, the fluorescence intensity of the PEG hydrogels increased with the increase of the aptamer concentration (Fig. 3E). These results clearly demonstrated that the acrydited aptamers were successfully incorporated into the PEG hydrogel network during the free radical polymerization.

3.3. Characterization of mechanical properties

It is important to understand the effect of the incorporated aptamers on the mechanical properties of the PEG hydrogels because the mechanical properties of a biomaterial play an important role in regulating cell behavior. The PEG hydrogels were characterized by the examination of their shear moduli and swelling ratio. Both storage (G′) and loss (G″) moduli of the aptamer-functionalized hydrogel were virtually the same as those of the native hydrogel (*i.e.,* the hydrogel without the aptamer functionalization) (Fig. 4A). When the sweeping frequency was varied in the range between 0.5 and 50 rad/s, the G′ values of the hydrogel functionalized with aptamers and the control hydrogel without any aptamers were both approximately 10,000 Pa. The swelling test showed that the hydrogels with and without the aptamers both absorbed water very quickly. The swelling ratio reached approximately 350% within the first hour but barely changed after 3 h (Fig. 4B). However, a difference in swelling was observed between the aptamer-functionalized PEG hydrogel and the native hydrogel though this difference was small (Fig. 4B). This result indicates that the incorporation of aptamers into the hydrogel network could, to some extent, change the mechanical properties of hydrogel, which was not revealed in the rheology analysis. To confirm this finding, the effect of aptamer concentration on hydrogel swelling was studied. The results showed that the swelling ratio increased when the concentration of the aptamer was increased (Fig. 4C). It is important to note that the swelling ratio increased by approximately 10% when the aptamer concentration was increased by two orders of magnitude (*i.e.,* from 2 μM to 200 μM) (Fig. 4C). Therefore, these results indicate that the chemical incorporation of aptamers with the concentrations used herein can only induce a moderate effect on the mechanical properties of the PEG hydrogels.

3.4. Examination of cell type-specific adhesion

In this proof-of-concept study, two cell lines were used as a model system, including CCRF-CEM and Ramos cells. The sgc8c aptamer was selected from a DNA library to bind CCRF-CEM cells with Ramos cells as a negative control [27]. Its binding affinity and specificity were previously investigated [26,27] and is further confirmed in this study by a flow cytometry assay. As shown in Fig. 5A, the sgc8c labeled CCRF-CEM cells exhibit stronger fluorescence intensity than the cells without any treatment or the control aptamer labeled cells. In addition, Fig. 5A also shows that the sgc8c treated Ramos cells (*i.e.,* the control cells) exhibit weak fluorescence intensity the same as that of the unlabeled cells. These results demonstrated that the sgc8c aptamer could specifically bind to the CCRF-CEM cells. After the binding capability of the sgc8c aptamer was determined, a cell adhesion test was performed to evaluate cell adhesion on the aptamer-functionalized PEG hydrogels. The results showed that a large number of CCRF-CEM cells could bind to the PEG hydrogel functionalized with the sgc8c aptamer (Fig. 5B). In contrast, very few CCRF-CEM cells were observed on the native PEG hydrogel and the hydrogel functionalized with the control aptamer. Quantitatively, the cell density on the sgc8c aptamer-functionalized hydrogel was ~850 cells/mm^2 whereas the average density was lower than 5 cells/mm^2 on the control hydrogels (Fig. 5C). In addition, consistent with the flow cytometry analysis, few Ramos cells adhered to the sgc8c aptamer-functionalized PEG hydrogel. To further characterize the cell adhesion to the aptamer-functionalized PEG hydrogel, a LIVE/DEAD cell staining assay was also used. Little cell death was observed on the aptamer-functionalized hydrogel during the cell adhesion experiment (Fig. 5D), indicating that the aptamer-functionalized

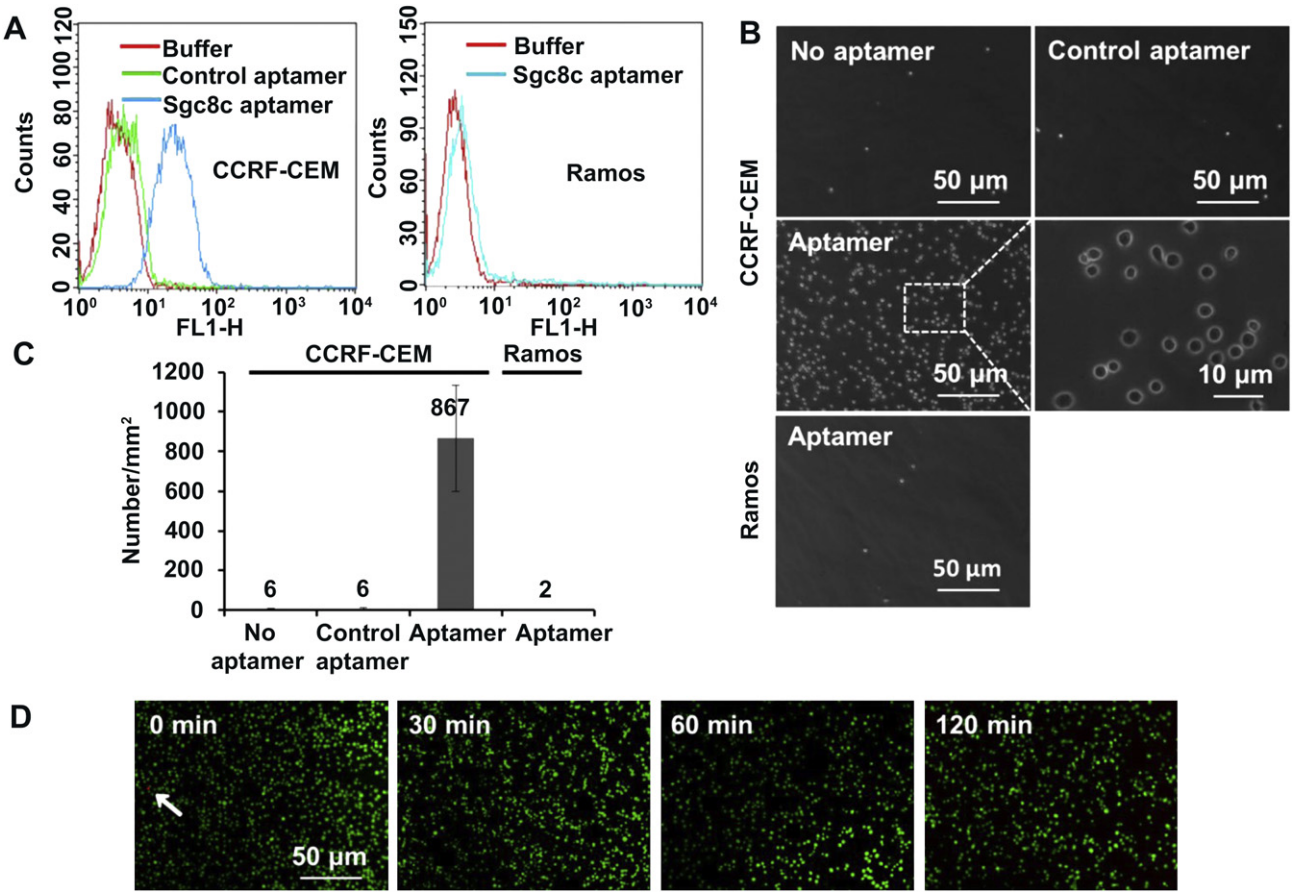

Fig. 5. Determination of cell type-specific adhesion on aptamer-functionalized PEG hydrogel. (A) Flow cytometry analysis of CCRF-CEM and Ramos cells labeled by sgc8c-FAM. (B) Representative microscopy images of cell adhesion. The concentration of the aptamer was 50 μM; the time of cell seeding was 30 min. (C) Quantitative analysis of cell adhesion. Three samples were prepared in each group and five different regions in each sample were randomly chosen. The cells were enumerated with the software Image J. (D) Staining of live/dead cells. Cells after the adhesion for a different period of time were stained with the mixture of calcein AM (green; live) and ethidium homodimer-1 (red; dead). The arrow indicates the dead cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PEG hydrogel was biocompatible. Therefore, these results showed that the PEG hydrogel without aptamers was resistant to cell adhesion whereas the incorporation of functional aptamers into the PEG hydrogel could successfully induce cell type-specific adhesion. In addition, the cells on the aptamer-functionalized PEG hydrogel could maintain viability after adhesion.

3.5. Effects of different parameters on cell adhesion

The effects of three parameters on cell adhesion were studied: the spacer length (*i.e.,* the adenosine tail of the aptamer), the concentration of the aptamer, and the time of cell seeding. Three aptamers with 0, 5, and 10As at their 5′ ends were used to prepare PEG hydrogels. The sgc8c-0A aptamer could induce cell adhesion. The cell adhesion induced by the sgc8c-5A aptamer and the sgc8c-10A aptamer was very similar. The data also showed that the cell density increased with the number of extra adenosines. In comparison to the sgc8c-0A aptamer, the sgc8c-10A aptamer could increase the average cell density on the hydrogel surface by ~ 50% (Fig. 6A). The density of the cells adhered to the hydrogel also increased with the concentration of the aptamer (Fig. 6B). The density increased from ~ 50 to ~ 850 cells/mm^2 with the increase of aptamer concentration from 2 to 50 μM (Fig. 6B). The increase of aptamer concentration over 50 μM did not lead to more cell adhesion. Similarly, the increase of seeding time resulted in the adhesion of more cells within 30 min, after which there was no significant increase in the number of adhered cells (Fig. 6C).

3.6. Attenuation of cell adhesion

To better demonstrate cell adhesion to the aptamer-functionalized hydrogel, additional two experiments were performed to attenuate cell adhesion. The first one was based on cell trypsinization. After a 15 min incubation of the cells in a trypsin solution, the cells were washed and treated with the FAM-labeled aptamers. The flow cytometry result showed that the trypsinized cells were still labeled by the FAM-labeled aptamers (Fig. 7A). However, the fluorescence intensity of the trypsinized cells was weaker than that of the intact cells. The mean fluorescence intensity was decreased by 55%. However, unlike the flow cytometry examination, the result of cell adhesion showed no significant decrease of cell adhesion (Fig. 7B). The second experiment was based on the treatment of the hydrogels rather than the cells. The PEG hydrogels were treated with COs that could effectively hybridize with the aptamers (Fig. 7D). The CO treatment led to the decrease of cell adhesion on the hydrogels (Fig. 7E). In addition, the number of cells decreased with the increased molar ratio of CO to aptamer. When the molar ratio of CO to aptamer reached 1:1, the cell density sharply decreased from ~ 800 to ~30 cells/mm^2 (Fig. 7E).

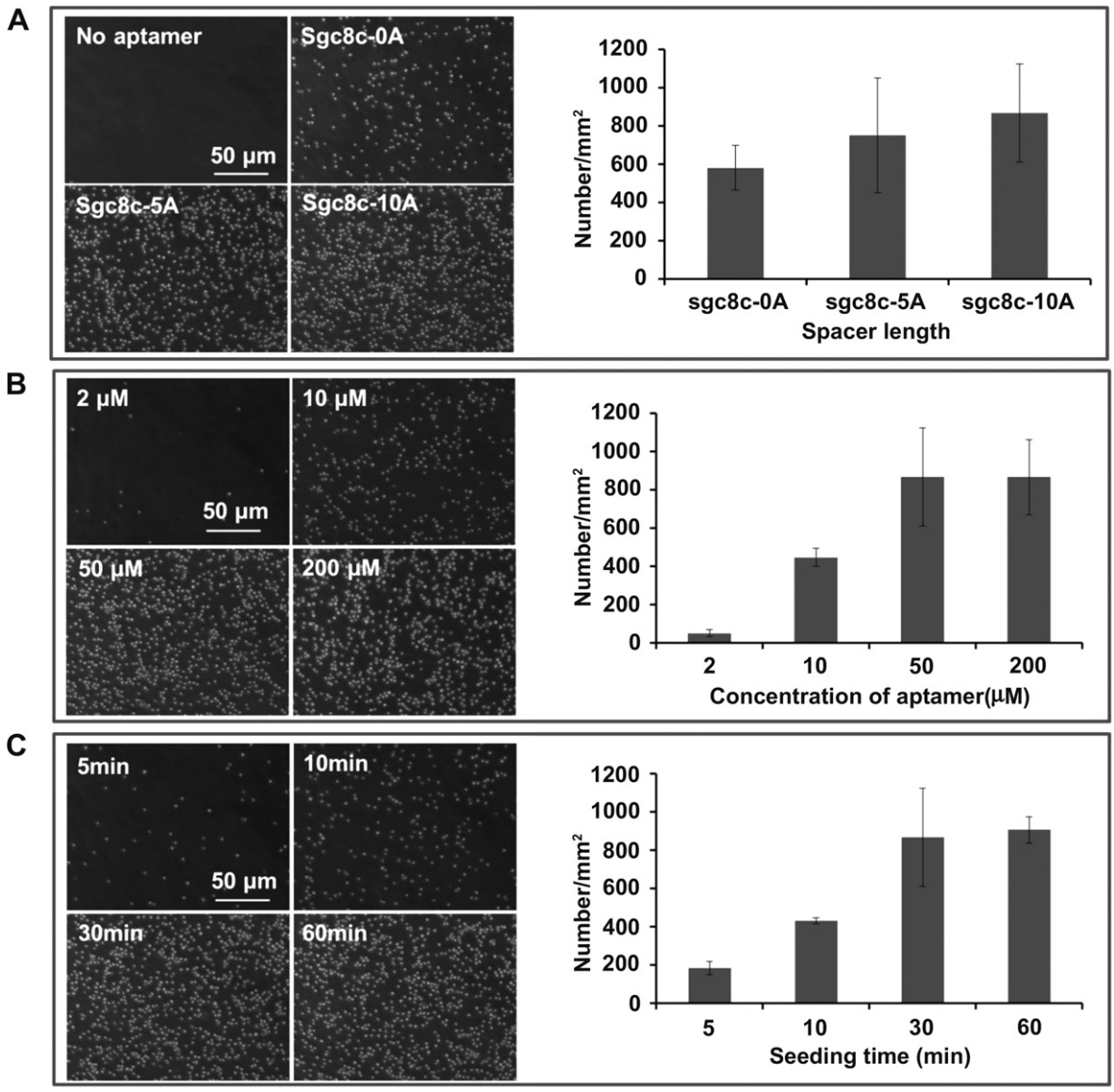

Fig. 6. Effect of different parameters on cell adhesion. (A) Effect of spacer length on cell adhesion. The concentration of the aptamer was 50 μM; the time of cell seeding was 30 min. (B) Effect of aptamer concentration on cell adhesion. The spacer length was 10A; the time of cell seeding was 30 min. (C) Effect of seeding time on cell adhesion. The concentration of the aptamer was 50 μM; the spacer length was 10A. Left: Representative cell images; right: Quantitative analysis of cell adhesion.

4. Discussion

Nucleic acid aptamers are an emerging class of affinity ligands that have been applied to the functionalization of numerous materials such as nanoparticles [28], titanium [29], and glass [30,31]. However, no study has been pursued to apply nucleic acid aptamers to functionalize hydrogels for developing an artificial extracellular matrix with dual function of controlling protein release and inducing cell adhesion. Our recent studies have shown that nucleic acid aptamers can be applied to hydrogel functionalization for controlled protein release [14–17]. Therefore, in this study, an aptamer-functionalized hydrogel model was developed with PEG and the sgc8c aptamer to study cell adhesion (Figs. 1 and 2).

The results showed that the aptamers could be successfully incorporated into the PEG hydrogel network through free radical polymerization (Fig. 3). However, it is also important to note that the polymerization was initiated by APS and TEMED. These two chemical reagents are not biocompatible. Therefore, a washing step was necessary to remove them from the PEG hydrogels before the cell adhesion tests. However, APS and TEMED based polymerization needs to be avoided for any *in situ* formations of hybrid cell-hydrogel constructs because these reagents can cause cell death during the mixing and polymerization procedures. This toxicity problem can be solved by using biocompatible methods of hydrogel synthesis such as photopolymerization [32]. Recent progress in photopolymerization has shown that bioactive molecules or mammalian cells can be incorporated into PEG hydrogels without the need of using substantially toxic initiators [33,34]. The synthesis of aptamer-functionalized PEG hydrogels using a biocompatible method is currently under our study.

Cell–material interactions can be affected by many properties of a biomaterial. One property is matrix rigidity [35–37]. The rheology analysis showed that the aptamers produced virtually no effect on the shear moduli (Fig. 4). Though the swelling test showed the increase of swelling when the PEG hydrogel was functionalized with the aptamers, the difference was small. This observation is

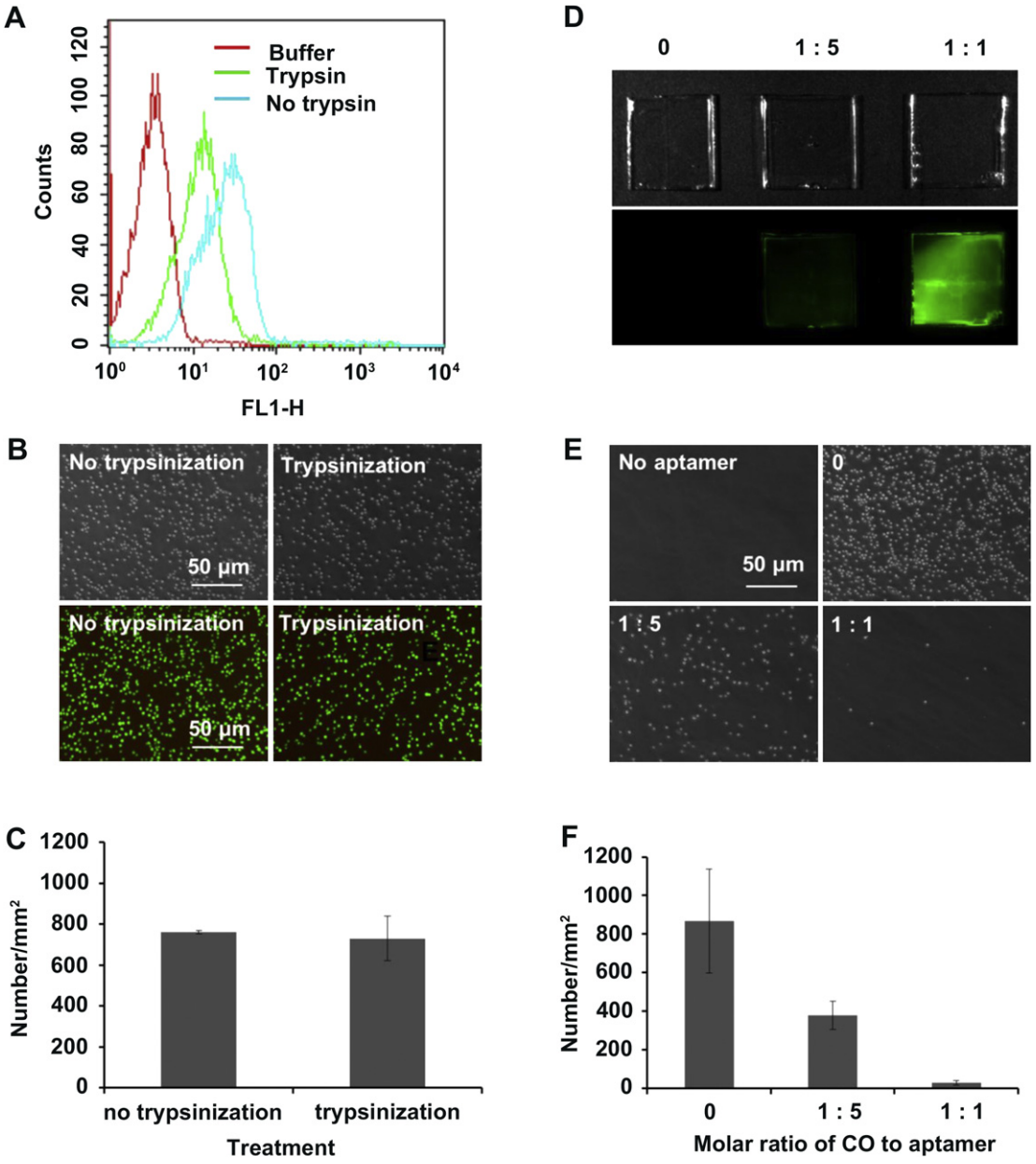

Fig. 7. Attenuation of cell-hydrogel interaction by trypsinization (A–C) and aptamer inactivation (D–F). (A) Flow cytometry analysis of cell trypsinization. (B) Images of cell adhesion (top) and live/dead staining (bottom). (C) Quantitative analysis of cell adhesion after trypsinization. (D) Fluorescence imaging of PEG hydrogel blocked with CO-FAM. The numbers show the molar ratios of CO to aptamer. (E) Representative images of cell adhesion. (F) Quantitative analysis of cell adhesion on hydrogel blocked with complementary oligonucleotides.

reasonable because the aptamers have a small molecular weight. In particular, the total amount of the aptamers used for developing the hydrogel herein is several orders of magnitude lower than that of PEG monomers. Therefore, though the incorporated aptamers may have an effect on their local PEG structure (*e.g.,* crosslinking density) during the hydrogel formation, the bulk properties of the hydrogel may not be significantly affected.

The cell binding results showed that the cells could adhere to the aptamer-functionalized PEG hydrogels whereas the native PEG hydrogel barely induced cell adhesion (Fig. 5). The observed resistance of cell adhesion to the native PEG hydrogel is consistent with previous reports [38–40]. In addition, little nonspecific cell binding to the aptamer-functionalized hydrogel and the control aptamer-functionalized hydrogel was observed. Thus, though matrix elasticity has been reported to affect cell binding [37], the cell adhesion to the PEG hydrogels must result from the incorporation of the functional aptamers. In addition, the results clearly show that nucleic acid aptamers can induce cell type-specific adhesion to a cell resistant hydrogel. This feature distinguishes aptamers from many adhesion ligands (*e.g.,* RGD peptides) that do not have binding specificity and can interact with a diverse array of cell receptors. When these ligands are used in hydrogel functionalization, multiple types of cells can randomly adhere on the hydrogel. The non-selectivity of cell adhesion may lead to the difficulty of organizing multiple types of cells in the specific locations of the same biomaterial to make an orderly, complex human tissue construct. Though only one aptamer model was used in this study, in principle, it is possible to chemically incorporate multiple

aptamers into the different locations of the same hydrogel. In this way, it is promising that multiple cells can be well organized to form biomimetic tissue constructs.

Because the spacer length of aptamer, the concentration of the aptamer, and the time of cell seeding are important factors to cell adhesion, they were studied to further understand aptamer-induced cell adhesion (Fig. 6). Previous studies showed that a long spacer between the hydrogel and the peptide ligands was necessary for effective peptide-induced cell adhesion [41]. Without the aid of a long spacer, the peptides may be buried in the hydrogel. As a result, they may not be available to cell receptors during the cell adhesion [41]. Unlike those results, the data presented here showed that the aptamers without the additional adenosine-based spacer could still induce cell adhesion to the PEG hydrogel (Fig. 6A). This difference between our finding and the literature is not unexpected. The affinity ligand used in this study was an oligonucleotide that forms a stem-bulge-loop structure (Fig. 2). In general, the stem of an aptamer is used to maintain its functional structure and may not directly participate in the binding between the aptamer and the target molecule [20]. The stem of the sgc8c aptamer contains eight base pairs that are approximately 2.7 nm in length. Thus, the stem itself plays a role as a spacer to extend the aptamers from the polymer backbone for cell contact. The result also showed the cell adhesion could be improved if extra nucleotides were added to the sgc8c aptamer (Fig. 6A), presumably because of the increased molecular flexibility and reduced steric hindrance. The cell adhesion was also enhanced with the increase of aptamer concentration and seeding time (Fig. 6B and C). Cell adhesion to a substrate is a dynamic process similar to a chemical reaction. The increase of concentration and time will increase the yield of the aptamer–receptor complexes, thereby inducing more cell adhesion. In addition, when the cell receptors are saturated or when the formation of the complexes reaches equilibrium, the increase of the concentration or time will not lead to the adhesion of more cells.

Besides the experiments designed to enhance cell adhesion to the PEG hydrogel, this study was also pursued to illustrate the potential of attenuating cell adhesion. In one experiment, cells were trypsinized prior to cell seeding. The reason for designing this experiment is three-fold. First, cells are often subjected to a multiple-step treatment before seeding. The density of cell receptors can decrease during the treatment. Second, receptor shedding, which can reduce the density of cell receptors, is common to many cells in normal physiological conditions. Third, cell harvesting from biomaterials is often used during the examination of cell–material interactions and the *ex vivo* expansion of specific cells (*e.g.,* stem/progenitor cells) [42]. As expected, the mean fluorescence density of trypsinized cells decreased by 55% (Fig. 7A), showing that the receptor density was significantly reduced by trypsinization. However, despite the decrease of the receptor density, the cell adhesion on the aptamer-functionalized PEG hydrogel showed no obvious change. This unexpected result showed that the aptamers could induce the adhesion of the same type of cells with a lower receptor density, presumably because of the high aptamer concentration and the high binding affinity of the aptamers. On the other hand, this finding also suggests that it might not be an effective strategy to attenuate cell–material interactions after cell adhesion by decreasing the density of cell receptors. In a second experiment, the aptamers in the PEG hydrogel were inactivated by their COs. After the aptamer inactivation, the cell adhesion to the aptamer-functionalized hydrogel was significantly decreased (Fig. 7E). Cell–matrix interactions are dynamic in nature [35]. The biomimetic materials that can provide the temporal control of cell–material interactions will provide more flexibility of regulating cell behavior at different time points when necessary. It has been found that the high density of affinity ligands on the substrate usually enhances cell adhesion [43]. However, the strong cell-substrate adhesion may subsequently inhibit cell migration and proliferation [44], which may not be beneficial for tissue repair or regeneration. Thus, the results of the aptamer inactivation experiment (Fig. 7E) suggest that aptamers and COs are a promising molecular recognition pair for the dynamic regulation of cell-hydrogel interactions.

5. Conclusions

An aptamer-functionalized PEG hydrogel was studied for determining the feasibility of using nucleic acid aptamers to mimic the adhesive binding sites of extracellular matrix. Nucleic acid aptamers can be successfully incorporated into the PEG hydrogel through free radical polymerization. In addition, aptamers can effectively induce cell type-specific adhesion to the PEG hydrogel. The level of cell adhesion can be altered by numerous parameters such as the aptamer concentration, the spacer length and the seeding time. Importantly, COs can block the binding functionality of aptamers in hydrogels and thereby attenuate cell adhesion in physiological conditions. In combination with our previous results showing that aptamers can control protein release from hydrogels with desired kinetics, the data presented in this study show that aptamer-functionalized hydrogels are promising biomaterials that can mimic the functions of extracellular matrices (*e.g.,* providing cells with biochemical and biophysical cues).

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