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Polymer Microneedle Mediated Local Aptamer Delivery for Blocking the Function of Vascular Endothelial Growth Factor

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Abstract

Overexpression of proteins in the body can cause severe diseases and other physiological disturbances. The development of protein blockers and local delivery systems would offer opportunities for addressing the health problems caused by protein overexpression. Nucleic acid aptamers are an emerging class of ligands with the potential to block proteins effectively; however, little effort has been made in developing polymer systems for local aptamer delivery. In this work, polymer microneedles capable of delivering DNA aptamers locally to inhibit the function of vascular endothelial growth factor (VEGF) were developed and studied. The presence of anti-VEGF aptamer in the polymer matrix did not change the apparent mechanical strength of the microneedles. Once in contact with a physiological solution, the polymer microneedles quickly dissolved, generating a high concentration of anti-VEGF aptamer in the surrounding local microenvironment. Aptamer delivery by way of dissolving polymer microneedles in a tissue phantom reduced VEGF-mediated endothelial cell tube formation. Thus, aptamer-loaded polymer microneedles hold great potential as a therapeutic tool for the treatment of human diseases resulting from protein overexpression.

For Table of Contents Use Only

Keywords

Protein overexpression; drug delivery; hydrogel; microneedles; aptamer

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Author Contributions

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1. Introduction

Proteins play a variety of critical roles in the human body. However, the quantity of these biomacromolecules in the body must be strictly controlled as their overexpression can cause severe human diseases^{1,2}. For instance, the overexpression of vascular endothelial growth factor (VEGF) has been shown to contribute to the development of cancer, the severity of psoriasis, and the pathology of age-related macular degeneration (AMD)^{1,3–7}. Thus, it is important to develop both drugs to block the overexpressed proteins and drug delivery systems to enhance the therapeutic effect.

A variety of molecules have been studied as candidates to block the bioactivity of proteins. Among these protein blockers, antibodies are the most commonly studied⁸. They are produced by plasma cells with high binding affinities and specificities against their corresponding target proteins^{9,10}. Antibody-based protein blocking has been widely studied in the treatment of diseases such as psoriasis, rheumatoid arthritis, multiple sclerosis, and many cancers^{11–14}. Despite the encouraging therapeutic effects observed in the clinic, antibodies as therapeutic agents have notable limitations^{15,16}. For instance, antibodies are produced biologically, which requires strict production control and causes batch-to-batch variation^{17,18}. They are also susceptible to irreversible denaturation which makes them hard to store on the shelf¹⁸. Antibodies also may present adverse immunogenic effects owing to their large sizes and production in biological systems¹⁸. Therefore, the development of antibody mimics as drugs has attracted great attention over the past two decades.

In addition to the development of antibody mimics, the advancement of drug delivery systems is an equally important mission for effectively inhibiting proteins. Hydrophilic polymers have been applied to fabricate a variety of matrices or reservoirs with or without crosslinking for the delivery of various drugs¹⁹. Because of their hydrophilicity and the mild conditions of preparing hydrophilic delivery systems in general, the bioactivity of biologics can be preserved at a relatively high level²⁰. Moreover, hydrophilic polymers can be dissolved or hydrolyzed in the body fluids²¹. Therefore, it is promising to apply hydrophilic polymers to develop dissolvable or biodegradable drug delivery systems for treating diseases related to protein overexpression.

The purpose of this work was to develop aptamer-loaded dissolving polymer microneedles and to examine aptamer function in blocking target proteins (Fig. 1). Nucleic acid aptamers are an emerging class of synthetic single-stranded oligonucleotides that have been studied as potential antibody mimics^{18,22–25}. While systemic aptamer delivery has been widely studied for the treatment of numerous human diseases^{26–28}, local aptamer delivery has not received as much attention. No report has demonstrated the development of dissolving microneedles for local aptamer delivery to block target proteins. Therefore, we fabricated aptamer-loaded polymer microneedles using the anti-VEGF aptamer as a model, examined their mechanical and dissolution properties, and evaluated their ability of blocking VEGF-mediated endothelial cell growth.

2. Materials and Methods

2.1. Materials

Sodium Acetate, sodium hydroxide, tween-20, polyvinylpyrrolidone (PVP, 10 kDa), and polyvinyl alcohol (PVA, 10 kDa) were purchased from Sigma Aldrich (St. Louis, MO). Medium 200 (M200), human umbilical vein endothelial cells (HUVEC), trypsin/EDTA, Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Extract, and Calcein AM were purchased from Life Technologies (Grand Island, NY). Agarose, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS), and fetal bovine serum (FBS) were obtained from Fisher Scientific (Pittsburgh, PA). Recombinant human vascular endothelial growth factor-165 (VEGF, $M_w = 38,200$ Da) was obtained from Peprotech (Rocky Hill, NJ). Polycarbonate microneedle master structures were purchased from Micropoint Technologies Pte (Singapore). All DNA aptamer sequences listed in Table 1 were purchased from Integrated DNA Technologies (Coralville, IA).

2.2. Methods

2.2.1. Prediction of Aptamer Secondary Structure—The secondary structure of the DNA aptamer was predicted using the program RNAstructure (<http://rna.urmc.rochester.edu/rnastructure.html>). The program is capable of predicting the structures of single- and double-stranded DNA or RNA. The predicted structure shown has the lowest free energy state.

2.2.2. Examination of Binding Affinity of Anti-VEGF Aptamer—The dissociation constant (K_D) of the anti-VEGF DNA aptamer was determined using surface plasmon resonance spectroscopy (SR7500DC; Reichert Analytical Instrument; Depew, NY) according to the method we reported previously²⁹. A carboxyl-functionalized sensor chip was used as the substrate to immobilize VEGF. Prior to VEGF injection, the surface of the chip was activated by the mixture of 0.05 g/mL EDC and 0.02 g/mL NHS at a flow rate of 20 μ L/min for 10 minutes. Subsequently, VEGF (10 μ g/mL in 10 mM sodium acetate, pH = 5.2) was injected for 10 minutes at 20 μ L/min for VEGF immobilization. The running buffer was PBS with 0.05% Tween-20. Before the test, the instrument was equilibrated with the running buffer for 30 min. During the test, the solution of anti-VEGF DNA aptamer flowed over the biosensor chip at 25 μ L/min for 6 min. Subsequently, the flowing aptamer solution was switched to the running buffer to obtain the dissociation profile. The biosensor chip was regenerated by washing with 30 mM NaOH at 100 μ L/min for 1 minute. Data were collected using SPR Autolink Software (Reichert Technologies), and plotted using Scrubber 2.0 (BioLogic Software). Briefly, the concentration data were zeroed and referenced by subtracting the reference channel signal. Kinetic analysis was performed by fitting a 1:1 biomolecular interaction model and plotting the maximum response. This enabled the association (k_a) and dissociation (k_d) constants to be determined and for the binding affinity (K_D) to be calculated.

2.2.3. Measurement of the tube formation of endothelial cells—80 μ L of Geltrex solution was added to each well of a 48-well cell culture plate and incubated at 37 °C for 30 minutes. HUVECs at 80% confluency were trypsinized and re-suspended in M200 supplemented with 0.5% FBS at 2.0×10^5 cells/mL. 200 μ L of the suspended HUVECs was

added to the Geltrex-coated wells and incubated for 30 minutes to allow cell attachment. Subsequently, the media was replaced with M200 containing anti-VEGF DNA aptamer and VEGF at a mole ratio of 1:1, 10:1, 100:1 or 1000:1, respectively. M200 with 0.5% FBS containing a scrambled anti-VEGF aptamer was used as a control. The cells were incubated at 37 °C in 5% CO₂ and 95 % relative humidity for 6 hours. After incubation, HUVECs were stained with 2 µg/mL Calcein AM for 30 minutes and imaged using an inverted fluorescence microscope (Olympus IX73, Center Valley, PA). The total tube length was quantified using ImageJ and the Angiogenesis Analyzer plugin. To calculate the inhibition efficiency, the following equation was used:

$$\text{Inhibition Efficiency (\%)} = \frac{T_+ - T_s}{T_+ - T_-} \quad 1)$$

where T₊, T₋, and T_s indicate the tube length of the positive control (with VEGF), negative control (no VEGF), and sample, respectively.

2.2.4. Microneedle Fabrication—Pyramidal master structures were obtained from Micropoint Technologies Pte, Ltd., Singapore. Each microneedle array consisted of 100 (10 × 10) pyramidal microneedles with a height, base, and tip-to-tip distance of 600 µm, 200 µm, and 500 µm, respectively. Female microneedles molds were made by casting PDMS into the master structures and allowing the PDMS solution to cure overnight at 37 °C.

The polymer microneedle arrays were fabricated according to a method published in the literature with slight modifications³⁰. In brief, 3 g of polyvinyl alcohol (PVA, Sigma Aldrich 10K) was mixed with 4 mL of water and heated to 90 °C for 2 hours. 1 g of polyvinylpyrrolidone (PVP, Sigma Aldrich 10K) was added into the PVA solution and the mixture was heated at 90 °C for another 2 hours. 5 µL of the PVA/PVP solution (20 % w/v) was cast into the PDMS mold to fabricate polymer microneedles. The PDMS mold was placed in a vacuum chamber for 2 minutes and centrifuged for 10 minutes at 3000 RCF (relative centrifugal force). Excess polymer was removed and the process was repeated 3 times to ensure all the microneedle cavities were full. After the microneedles were dried for a half-hour, 50 µL of the PVA/PVP solution (50 % w/v) was cast on the top of the microneedle array as a base plate and dried for 48 hours to form the final microneedle array.

To fabricate microneedles containing FAM-labeled DNA aptamer (Table 1.) for the distribution assay, 5 µL of polymer solution containing 1 mM of FAM-aptamer was mixed with 10 µL of 30 % w/v PVA/PVP solution and cast into the microneedles tips in the same manner as described above. To fabricate microneedles containing anti-VEGF DNA aptamer, the aptamer was mixed with the PVA/PVP solution. The final polymer solution was 20 % w/v solution and the microneedles were fabricated following the same procedure as described above.

2.2.5. Characterization of the Morphology of Microneedle Arrays—The morphology of microneedle arrays was characterized using scanning electron microscopy (FEI NOVA NanoSEM 630 FESEM), optical profilometry (Zygo Nexview 3D, United

States), and stereomicroscopy (Olympus MVX10, Center Valley, PA and Olympus BX61, Center Valley, PA).

2.2.6. Examination of the Dissolution of Microneedle Arrays—To determine the dissolution of the polymer microneedles, 66 μL of agarose (1% in PBS) was cast into a cylindrical mold and cooled to form an agarose disk with dimensions of 5 mm $\times$ 2 mm in radius and thickness, respectively. The polymer microneedle arrays were inserted into the agarose disk. After a predetermined time interval (15, 30 or 45 seconds), the polymer microneedle arrays were removed and immediately placed in an 80 $^{\circ}\text{C}$ oven to dry the surface of the microneedles before imaging. The microneedles were imaged under an Olympus MVX10 microscope (Center Valley, PA) and quantified using ImageJ.

2.2.7. Measurement of the Mechanical Strength of Microneedle Arrays—The mechanical strength of microneedles with and without anti-VEGF DNA aptamer was evaluated using an axial compression instrument (Instron 5960, Norwood, MA). A movable head equipped with a 10N load cell was preset to a maximum extension of 600 μm and a compression rate of 1.1 mm/s before delivering an axial force perpendicular to the microneedle array. The applied force was measured from the point of contact until needle failure, a point characterized by a discontinuity or plateau observed using Bluehill[®] software. The resultant stress-strain relationship was plotted and the average fracture force per microneedle was calculated from the maximal fracture force divided by the total number of microneedles in the microneedle array.

2.2.8. Examination of Microneedle Mediated Aptamer Delivery in the Tissue Phantom and the Surrounding Microenvironment—Agarose hydrogel was used as a tissue phantom. 66 μL of 1 % agarose solution was cast into a transwell insert and allowed to cure for 30 minutes at 4 $^{\circ}\text{C}$. Subsequently, a microneedle array containing FAM-labelled anti-VEGF aptamer was inserted into the agarose hydrogel and the transwell was placed into a well of a 24-well plate containing 560 μL of PBS. After 20 minutes, the microneedle array was removed and the transwell and well were imaged using a CRI Maestro Imaging System (Cambridge, MA). The exposure time for the transwell and well were 25 ms and 100 ms, respectively. We used a longer exposure time for the well because the aptamer was very dilute in the well. The 24-well plate was incubated at 37 $^{\circ}\text{C}$ in a humid atmosphere with 5 % CO_2 and imaged hourly for 6 hours.

2.2.9. Evaluation of the Function of Aptamer-Loaded Polymer Microneedles in Mediating the Inhibition of Tube Formation—160 μL of the Geltrex solution was added to each well of a 24-well cell culture plate and incubated at 37 $^{\circ}\text{C}$ for 30 minutes to allow the Geltrex gel to solidify. 66 μL of the agarose solution (1%) was placed into a transwell insert. Prior to the gelation of the agarose solution, 7 ng of VEGF was quickly mixed with the agarose solution and the insert was immediately placed in 4 $^{\circ}\text{C}$ for 30 minutes to allow gel solidification to ultimately form a VEGF-containing tissue phantom. 400 μL of the suspended HUVECs (2.0×10^5 cells/mL) was added into the Geltrex-coated wells and incubated for 30 minutes to allow cell attachment. Subsequently, the medium was replaced with M200 supplemented with 0.5% FBS. Microneedles containing 2 pmol, 20

pmol, or 200 pmol of anti-VEGF DNA aptamer were inserted into the tissue phantom on the transwell insert for 30 minutes. The transwell insert was placed in the well containing HUVECs. Microneedles containing a scrambled anti-VEGF DNA aptamer were used as a control. The cells were incubated at 37 °C in 5% CO₂ and 95 % RH for 6 hours. After incubation, HUVECs were stained with 2 µg/mL Calcein AM for 30 minutes and imaged using an inverted fluorescence microscope (Olympus IX73, Center Valley, PA). The total tube length was quantified using ImageJ.

2.2.10. Statistical Analysis—Quantitative data are expressed as mean ± standard deviation of the mean. For statistical analysis of multiple groups, one-way ANOVA with Tukey's test was performed with p-value = 0.05 using Minitab 17 statistical software (State College, PA). Student's t-test was used when comparing two groups. The result is considered statistically significant when p-value = 0.05.

3. Results and Discussion

Evaluation of Aptamer Mediated VEGF Inhibition

In this work, we used an anti-VEGF aptamer as a model to demonstrate the therapeutic potential of local aptamer delivery (Fig. 2A). The bioactivity of VEGF or any other overexpressed protein can in principle be blocked with small molecules, peptides, soluble decoy receptors and antibodies^{16,31}. Small molecules or peptides can be chemically synthesized with relatively low costs. However, their binding specificity and affinity are generally low, which can cause off-target effects. Soluble decoy receptors and antibodies are large biomolecules with substantially higher affinity and binding specificity compared to smaller molecules. However, not only is the manufacturing process of these molecules cost prohibitive, but the products generally exhibit low stability. Different from traditional protein blockers, aptamers are single-stranded nucleic acids that are screened from synthetic nucleic acid libraries^{32,33}. Aptamers are small in size with usually 25 to 40 nucleotides (nt). Owing to their short sequences, they can be synthesized using standard chemical reactions with a much lower manufacturing cost than decoy receptors and antibodies. They are also stable and well tolerant of harsh manufacturing conditions and environmental fluctuation. They can discriminate targets on the basis of subtle structural differences such as the presence or absence of a methyl or a hydroxyl group in certain proteins³⁴. Thus, aptamers hold great potential as antibody mimics and protein blockers to treat human diseases resulting from protein overexpression.

The model aptamer used herein was an anti-VEGF aptamer that was originally selected with the SELEX protocol by Tuerk et al³⁵. The sequence and structure of its truncated form are shown in Table 1 and Fig. 2A. It has 26 nucleotides (nt) and its secondary structure has a loop, a stem, and two tails hanging at each end of the aptamer. As its binding capability was not well characterized previously, we used SPR to examine its K_D value. We varied the concentration of the aptamer from 25 nM to 1 µM and acquired a series of profiles showing aptamer association and dissociation (Fig. 2B). By correlating the SPR response and the concentration (Fig. 2C), we were able to calculate the association rate constant (k_a ; 2.69×10^4

$\text{M}^{-1} \text{s}^{-1}$), dissociation rate constant (k_d ; $2.65 \times 10^{-3} \text{s}^{-1}$), and dissociation constant (K_D ; 99 nM). These data suggest that this aptamer has a decent binding affinity for VEGF.

As the aptamer used in this work was a DNA aptamer, one concern with its use was its stability in biological fluids. Thus, we examined the stability of the aptamer in the cell culture medium used in the tube formation assay. After the treatment of the aptamer with the cell culture medium, gel electrophoresis was carried out to determine the extent of degradation (Fig. 2D). We initially expected that the unmodified aptamer would degrade and the modified aptamer could exhibit high stability. However, the results showed that the aptamer with or without modifications did not exhibit significant degradation in the current in vitro working condition. The difference between the results and the initial expectation should stem from the low concentration of serum used in this work. For the tube formation assay, the serum concentration was only 0.5% and thus the amount of nucleases in the cell culture medium might be too low to cause any obvious degradation of unmodified DNA aptamers. To increase the stability of nucleic acids, numerous chemical methods have been developed for modifications^{36–39}. These methods include both end and internal modifications. Both methods have been successfully used to increase the stability of nucleic acid aptamers. For instance, aptamers composed entirely of 2'-O-methyl nucleotides did not exhibit any detectable degradation after incubation in plasma for 96 h at 37 °C⁴⁰.

VEGF is the major angiogenic growth factor for stimulating angiogenesis, during which endothelial cells can grow, sprout and assemble⁴¹. This capability can be represented in vitro by using VEGF to stimulate the tube formation of endothelial cells on a Geltrex-coated surface. Thus, to assess the ability of the anti-VEGF aptamer to block VEGF, the endothelial cell tube formation assay was conducted. We added the anti-VEGF aptamer into the cell culture medium with a final concentration of 25 nM (100:1 aptamer to VEGF mole ratio) due to the modest binding affinity of the aptamer as indicated by the K_D value. The results showed that the anti-VEGF aptamer could effectively inhibit the tube formation of HUVECs in comparison to the control groups (Fig. 3A), suggesting that VEGF was bound and blocked by the aptamer. We further varied the amount of the aptamer in the cell culture medium. The concentration of the aptamer was varied from 0.25 to 250 nM (1:1 to 1000:1 aptamer to VEGF mole ratio). The results showed that the tube formation was not significantly inhibited in the 1:1 group (Fig. 3B&C). This result is reasonable as the entire system involved three molecules rather than only aptamer and VEGF. Other molecules include the VEGF receptors that have high binding affinities for VEGF on the nanomolar level and can compete against the aptamer for binding to VEGF. Thus, the concentration of the aptamer must be increased to improve the ability of the aptamer to inhibit VEGF. Indeed, when the concentration reached 2.5 nM (10:1 aptamer to VEGF mole ratio), the inhibition efficiency was increased to over 75% (Fig. 3B&C). The inhibition efficiency further increased to nearly 100% and reached a plateau when the ratio of aptamer to VEGF was increased to 100:1 (Fig. 3B&C).

Fabrication and Characterization of Aptamer-Loaded Polymer Microneedles

Protein blockers can be delivered systemically to block the activity of overexpressed proteins in a certain tissue. However, it is inevitable for drugs to reach normal tissues

expressing the same protein at a standard level, which can cause side effects. Moreover, to reach the target tissue, drugs have to overcome physiological transport barriers that can significantly limit drug delivery. As a result, a large dose is often required to achieve effective treatment. Local drug delivery avoids all of these issues. Microneedle technologies offer the opportunity for successful local drug delivery to numerous external tissues such as the skin and the eye^{42–44}. Hypodermic injection of particles or in-situ forming implants can, in principle, achieve the same therapeutic effects as microneedle technologies. However, these prominent systems for local drug delivery may have low patient compliance and heterogeneous drug distribution, which are not concerns for microneedle technologies. Thus, microneedles have been extensively studied for local drug delivery^{45,46}.

The development of microneedles for local delivery of therapeutic aptamers has not been previously explored before. Thus, after showing that the aptamer could block VEGF bioactivity, we proceeded to fabricate polymer microneedles for the anti-VEGF aptamer. Polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) were used to fabricate the microneedle array. PVP was initially used as a plasma volume expander for trauma victims and is a component of many drug formulations approved by the U.S. Food and Drug Administration (FDA)^{47,48}. PVA has been applied to many biomedical applications including wound dressings, vascular grafts, and a tissue scaffold^{49–51}. These two polymers have been studied for the preparation of microneedles in vaccination owing to their biocompatibility^{52,53}. Thus, we chose to apply PVP and PVA to develop dissolving microneedles for aptamer delivery in this work. Through a double-casting process we successfully fabricated a 10×10 polymer microneedle array (Fig. 4A). Upon closer examination, the pyramidal microneedles were 600 μm in height with a base width and tip diameter of 200 μm and 10 μm , respectively (Fig. 4B–D). These measurements are consistent with the geometry of the PDMS mold. We used this geometry because it provides a small aspect ratio that has been shown previously to enhance mechanical strength and increase insertion capability^{54–56}. Notably, a 20 % PVA/PVP solution was used to prepare the microneedle tips because the polymer mixture at this concentration was still able to be easily pipetted owing to a reasonably low viscosity. After the needle tips were dried, a high viscosity PVA/PVP solution was used as the backing layer to support the microneedle tips. The increased solution viscosity ensures drug localization in the needle tips by reducing diffusion³⁰.

To ensure adequate aptamer delivery, the microneedles need to be dissolvable or degradable in aqueous solutions. Both PVA and PVP are highly hydrophilic³⁰. To evaluate the dissolution of their composite microneedles, we inserted the PVA/PVP microneedle array into an agarose hydrogel that was used herein as a tissue phantom. Within 15 s, the height of the microneedles decreased by 70% (Fig. 5A). After 30 s, the tip regions of the microneedles seemingly disappeared completely with only the microneedle bases remaining. These results demonstrate that the aptamer-loaded PVA/PVP microneedles are highly dissolvable. While the dissolution of the PVA/PVP microneedles is fast, the time for dissolution can be adjusted by using other hydrophilic polymers (e.g., hyaluronic acid).⁵⁷

As mechanical strength is critical to microneedles in real applications, we examined whether the presence of aptamers in the microneedle matrix would cause any effect on the

mechanical strength of microneedles. The tip regions of the microneedles bent after a force normal to the microneedle array was applied until a 300 μm compressive force corresponding to ~ 5.6 N. Tip-bending was determined to be the mode of failure, instead of compression or fracturing, based on the stress-strain profiles. Tip bending resulted in a shoulder on the stress-strain curve⁵⁸. The stress-strain profiles show that the PVP/PVA microneedles and the aptamer-loaded PVP/PVA microneedles had a fracture force at 0.054 N/needle and 0.053 N/needle, respectively (Fig. 5B). The necessary force for a microneedle of 25 μm in diameter is approximately 0.058 N/needle^{59,60}. Since our microneedle tip diameter is between 5 and 10 μm , the necessary force for skin insertion may be reduced. However, it needs to be validated in the future in vivo test. The results also showed that these stress-strain profiles nearly overlapped, demonstrating that the presence of aptamers in the microneedles did not affect the mechanical strength of the microneedles.

Aptamer-Loaded Polymer Microneedles for Blocking VEGF-Mediated Tube Formation

To illustrate the potential of microneedle-mediated local aptamer delivery, we fabricated microneedles with the FAM-labeled aptamer (Fig. 6A). The microneedles were inserted into the agarose gel that was formed in the transwell insert. After the transwell insert was placed into the 24-well plate, the agarose gel and the solution in the well were imaged hourly to assess the aptamer release and dissipation. Immediately after insertion, the FAM-labelled aptamer was confined in the center of the agarose gel (Fig. 6B). With the increase of time, the fluorescence intensity in the original location of microneedle insertion started to gradually decrease. This observation indicates the dissolution of microneedles and the release of FAM-labeled aptamers, which is consistent with the data shown in Fig. 5. At 6 h, the fluorescence intensity in the tissue phantom decreased by $\sim 80\%$. Meanwhile, the fluorescence intensity of the solution in the well linearly increased with time (Fig. 6C). Taken together, these results demonstrate that the aptamers in the PVP/PVA microneedles could be released after the dissolution of the microneedles and further dissipated in the surrounding microenvironment.

As the ultimate goal of this research is to examine whether the aptamer-loaded dissolvable microneedles can block protein bioactivity in a diseased tissue, we loaded VEGF into the agarose gel to mimic a diseased situation with an overexpressed target protein and inserted the aptamer-loaded microneedles into the agarose gel. The results demonstrated that the ability of the VEGF-loaded agarose gel in stimulating the tube formation was significantly inhibited by the aptamer-loaded PVP/PVA microneedles (Fig. 7). There are two possible reasons for the aptamer-mediated VEGF inhibition. First, as shown in Figs. 6 B and C, during the 6 h treatment, $\sim 80\%$ of aptamers were released from the agarose gel and $\sim 20\%$ of aptamers were still entrapped in the agarose gel. This observation might be due to the physical binding of aptamers to the agarose hydrogel, which slowed down the diffusion of aptamers to the well. The presence of aptamers inside the agarose gel would sequester a significant amount of VEGF within the agarose gel and reduce the concentration of VEGF in the well. This possibility would benefit the treatment of human diseases since overexpressed proteins would be confined and inhibited locally by aptamers. Second, while aptamers and VEGF could depart the agarose gel, the stable aptamer-VEGF complexation would not allow the presence of a large amount of free VEGF in the well for binding to the cell receptor.

While we have demonstrated the success of using aptamer-loaded dissolving microneedles to block the VEGF bioactivity in stimulating tube formation, this technology could be further improved if this aptamer had higher binding affinity similar to soluble decoy receptors and antibodies that bind proteins with a K_D value lower than 1 nM or even 100 pM³¹. The anti-VEGF DNA aptamer was selected from the traditional DNA library and is made of natural nucleotides. Recent advances in aptamer selection have shown that aptamers can be modified to acquire protein-like properties^{61,62}. By adding functional groups to nucleotides to mimic amino acid side-chains, it is possible to expand the chemical diversity of aptamers and select aptamers with high affinities similar to those of decoy receptors and antibodies. For instance, Larry *et al* selected DNA aptamers over 1000 human proteins from chemically modified libraries that incorporated dUTPs modified at the 5' position⁶³. A majority of these aptamers had K_D values lower than 1 nM and many of them had K_D values lower than 100 pM. Using these high affinity aptamers to develop microneedles is expected to further improve the therapeutic potential of the microneedles by reducing the amount of aptamer loading and improving the effectiveness of protein blocking.

4. Conclusion

In summary, the data have successfully demonstrated the development of dissolving polymer microneedles for local aptamer delivery. The microneedles containing aptamers maintained high mechanical strength and can be quickly dissolved under physiological conditions to release the loaded aptamers to the surrounding microenvironment. Moreover, the released aptamers can block the activity of protein and inhibit protein-mediated cell growth. Therefore, this aptamer-microneedle system holds great potential for blocking overexpressed proteins and treating human diseases such as psoriasis, skin cancer and macular degeneration.

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ABBREVIATIONS

Apt	aptamer
VEGF	vascular endothelial growth factor
sApt	scrambled aptamer

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Figure 1.

Schematic illustration of the concept. (A) After the insertion of microneedles into the target tissue, the aptamer will be released from the dissolving microneedles to block the overexpressed protein. (B) Protein blocking occurs via intermolecular complexation between protein and aptamer.

Figure 2. Characterization of the anti-VEGF aptamer. (A) Prediction of secondary structure. (B) Kinetic analysis of aptamer binding to immobilized VEGF (top). Relationship of maximal SPR response and the aptamer concentration (Bottom). (C) PAGE gel image showing the stability of the aptamer. Lane 1: Apt; lane 2: Apt incubated in M200 supplemented with 0.5% FBS; lane 3: Mod-Apt; lane 4: Mod-Apt incubated in M200 supplemented with 0.5% FBS. The amount of Apt or Mod-Apt was 10 pmol.

Figure 3.

Examination of the blocking ability of the aptamer. (A) Comparison of different treatments in inhibiting the tube length of HUVECs. sApt: scrambled aptamer. All aptamers were added directly to the media containing VEGF (10 ng/mL). The numbers indicate the mole ratios of aptamer to VEGF. ***, $P < 0.001$. (B) Effect of the amount of the aptamer on the inhibition efficiency. (C) Representative images of cell tubes formed in different conditions. The concentration of VEGF was 10 ng/mL. The numbers indicate the mole ratios of aptamer to VEGF. Scale bar: 200 μm. Cells were stained with Calcein AM, imaged under a fluorescent microscope and quantified.

Figure 4. Characterization of the morphology of polymer microneedles. (A) Photograph of the microneedles. Scale bar: 1 mm. (B) Optical image of a single polymer microneedle. (C&D) SEM images of the polymer microneedles from the top (C) and the side (D) view. Scale bar: 1 mm. Inset is a zoomed-in SEM image of a single polymer microneedle (scale bar: 100 μm).

Figure 5. Examination of the dissolution and mechanical strength of the polymer microneedles. (A) Images of polymer microneedles at different time points after insertion into a tissue phantom. From top to bottom: 0 s, 15 s, 30 s, and 45 s, respectively. Remaining microneedle height was quantified using ImageJ. Scale bar = 200 μm . (B) Mechanical behavior of the polymer microneedles. The two representative images show the morphology of the microneedles before (left) and after (right) compression. Scale bar = 200 μm . Force per microneedle was recorded as a function of plate extension culminating in microneedle

fracture (as indicated by $\blacklozenge$). Fracture force per microneedle was 0.053 ± 0.004 N and 0.053 ± 0.006 N for microneedles with and without aptamer, respectively.

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Figure 6.

Examination of aptamer release in the tissue phantom and the surrounding microenvironment. (A) Fluorescence image of polymer microneedles containing FAM-labelled DNA aptamer. Scale bar = 200 μm . (B) Representative fluorescence images of the tissue phantom and media after microneedles were inserted. Top: images of tissue phantom at 0, 3 and 6 h post the insertion. Bottom: images of the surrounding media at 0, 3 and 6 h post the insertion. (C) Fluorescence intensity in the tissue phantom and media at the different time points.

Figure 7. Examination of microneedle-mediated aptamer delivery for the inhibition of tube formation. (A) Effect of different treatments on the inhibition efficiency. sApt: scrambled aptamer. *, P 0.05. (B) Representative images of the tubes formed in response to different treatments. Scale bar = 200 μ m.

Table 1

List of aptamer sequences.

Aptamer	Aptamer ID	Sequence (5'-3')	Length (nt)	Molecular Weight (Da)
anti-VEGF aptamer	Apt	CCC GTC TTC CAG ACA AGA GTG CAG GG	26	7,981
End-modified anti-VEGF aptamer	Mod-Apt	/56-FAM/CCC GTC TTC CAG ACA AGA GTG CAG GG/3InvdT/	26	8,822
scrambled anti-VEGF aptamer	sApt	GTG TTAACG AGG CTC GCA CCG GAC AC	26	7,981