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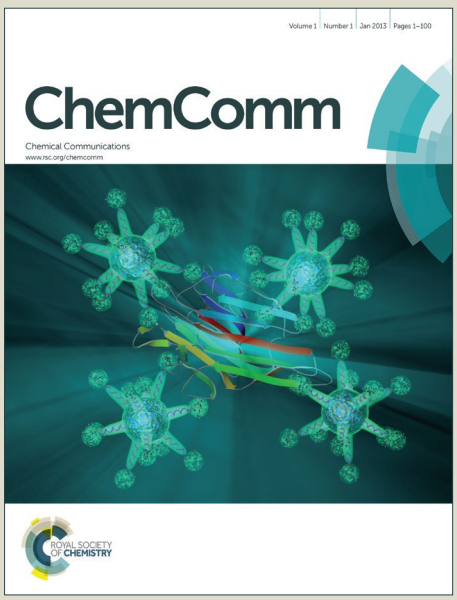

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Switchable Probes: pH-Triggered and VEGFR2 Targeted Peptides Screening through Imprinting Microarray

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One switchable affinity peptide STP is screened out from a high-throughput library by an integrated imprinting microarray. STP is pH triggered and also the ligand of tumor marker VEGFR2. Efficient cell recognition and penetration as well as *in vivo* image could be "turned on" and accelerated only in the condition of VEGFR2 overexpression and mild acidic environment.

Tumor developing process cause the pathological imbalance of transformed cells with their surrounding environments and result in the formation of acidic tumor microenvironment (TME),[1] which associate with tumorigenesis, invasion and metastasis.[2] Recent progress reveals that by understanding the internal mechanism of the TME, various chemical agents for diagnosis and therapy have been developed.[3, 4] In another aspect, angiogenesis is the pathway which is responsible for most blood vessel growth during development of tumors.[5] If angiogenesis is prevented, tumor growth could be prevented to a large extent. Vascular endothelial growth factor receptor 2 (VEGFR2/KDR/Flk-1), a 150-kDa transmembrane protein is one of the angiogenesis markers and is recognized as an attractive target for cancer diagnosis and therapy.[6] Traditionally, antibodies are the most common targeting agents for the recognition towards specific VEGFR2.[7] However, the large size of antibodies restricts their penetrating ability and nonspecific uptake by the mononuclear phagocyte system.[8] It is of great important to develop small molecule probes with high affinity and specificity towards VEGFR2 and equal importance for the molecule probes to recognize both the acidic TME. As excellent small molecules, peptides own many advantages such as good-penetrability, low-immunogenic and low-toxicity. Recent studies show some peptides have been proved to recognize the specific acidic microenvironment [9] and identify the tumor markers.[10] Furthermore, with the development of the MEMS (micro electro mechanical system) techniques, small molecule screening could be realized in microarray chips with the high-throughput, integration and *in situ* detection features by surface modification of the detecting spots in the array.[11, 12] The microarray strategy has developed into a powerful biochemical tools which is further applied in molecular recognition and diagnosis.[13]

Based on our previous work,[14, 15] we report herein a switchable peptide probe towards both VEGFR2 and TME. The dual recognition peptide was *in situ* screened out from a high throughput combinatorial chemistry library through an integrated imprinted microarray device (Scheme 1 and Figure S1, Electronic Supplementary Information ESI). One-bead–one-compound (OBOC) approach was employed to construct a 10^6 peptide library towards VEGFR2 and peptide screening in acidic condition was achieved (Figure S2). In the library, Methionine was used for assisting *in situ* chemical cleavage and Cysteine was used to provide the thiol group. Through the biotin-streptavidin (SA) conjugation[16] (Figure S3) and magnetic beads assisted screening (Figure S4), about 400 positive beads were isolated from the library. Silver-sputtered chip with microwell array could trap the positive beads in one-well-one-bead manner. Peptides on beads were *in situ* cleaved. Part of the peptide in each well was *in situ* released onto a bear gold chip to print the peptide array through the S-Au bond. The gold surface chip was for affinity analyses by Surface Plasmon Resonance imaging (SPRi) (Figure S5 and S6), and peptide left in the silver-sputtered chip was remained for single bead sequencing by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) (Figure S7). In this way, the sequences and the affinities of the positive peptides could be obtained simultaneously. We got the conserved peptide sequence of SKDEEWHKNNFPLSP (STP) for acidic environment and TIDHEWKKTSFPLSF (TP) for neutral environment with nanomolar affinity towards VEGFR2 (K_D of STP was 8.50×10^{-8} M in acidic condition and K_D of TP was 5.93×10^{-7} M in neutral condition). Between the two peptides, STPhad both VEGFR2 targeting and pH-dependent properties.

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Electronic Supplementary Information (ESI) available: [Some experimental details, additional figures are included in the ESI].

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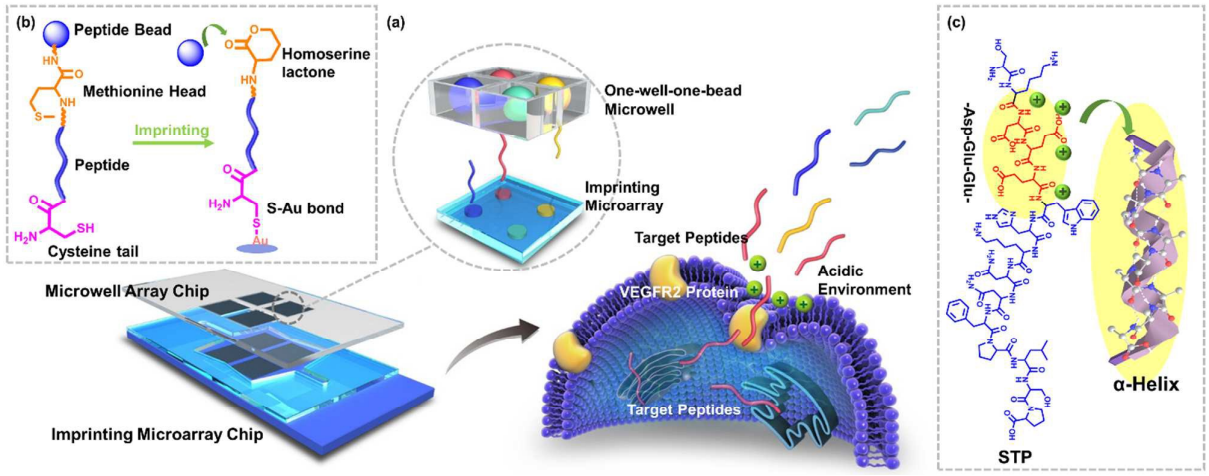

Scheme 1. Schematic diagram of integrated OBOC peptide library screening device and illustration of dual-functional pentadecapeptide with pH dependent function. (a) The integrated imprinted microarray of which peptide could be detected with both the SPRi and MALDI-TOF-MS. (b) The imprinting process from the microwell array to the SPRi array (c) pH dependent peptide could penetrate into the cells because of the formation of the α-helixes.

Then we tested the binding specificity of STP and TP toward VEGFR2 protein in the living cells, the VEGFR2 high-expressing human umbilical vein endothelial cell line HUVEC was chosen as VEGFR2 overexpression cell model. HUVEC was reported to have specific binding sites (VEGFR2, with 500 sites/cell) of VEGF and the dissociation constant was reported as low as 9 pM.[17] Human embryonic kidney cell line 293T with none expression of VEGFR2 served as the negative cells. STP and TP were de novo synthesized and labeled with fluorescein isothiocyanate (FITC, green channel) (Figure S8 and S9). To further confirm the specificity of the peptides, co-localization experiment was also performed. Phycoerythrin (PE, red channel) labeled anti-human VEGFR2, a positive VEGFR2 indicator was employed. The PE fluorescent intensity was shown in red channel. As shown in Figure 1a-h, the red and green fluorescent signals were almost overlapped. It was indicated that the antibody's binding behavior was the same as the peptides. Afterwards, we tested the targeting and pH-dependent properties of STP towards VEGFR2. As expected, FITC-STP preserved the binding ability toward HUVEC cells on the membrane only at pH 5.8 (Figure 1i-p) while FITC-TP showed binding affinity both at both pH 5.8 and pH 7.4 (Figure 1q-x). For quantitative comparison, fluorescence intensity profiles along through the red rows were also determined. It showed that the binding behavior of STP towards HUVEC was quite different in different pH conditions while the one of TP was almost unchanged. It was obviously that the STP showed pH-triggered ability. For negative cells, none of the peptides showed binding to the cell surface (Figure S10). Additionally, we tested the toxicities of the peptide probes by MTT assay and found these peptides might be safely target the VEGFR2 with a very low toxicity (Figure S11).These results confirmed that STP and TP showed good specificity toward VEGFR2 in their specific microenvironment, respectively. What's worth mentioning was that the fluorescent intensity of the antibody in acidic environment was lower than that in neutral environment, which may due to that the acidic microenvironment was not suitable for antigen-antibody recognition while our peptide STP showed satisfactory binding affinity in the acidic microenvironment. We considered STP as a dual recognition peptide probe and supposed that the pH dependent characteristic of STP was relevant to the isoelectric point (pI) of the peptide. The pI of STP (SKDEEWHKNNFPLSP) was calculated as 5.4 and the pI of TP (TIDHEWKKTSFPLSF) was 7.5. In the neutral environment, ionization would not happen for TP. While in the acidic environment, TP would be positive charged which could show binding behavior towards the negative cell membrane. However, STP would be negative charged in the neutral environment, which would show electrostatic repulsive force against the cell membranes. Therefore, the activity of STP could be "turned on" only in the existence of VEGFR2 in the acidic condition.

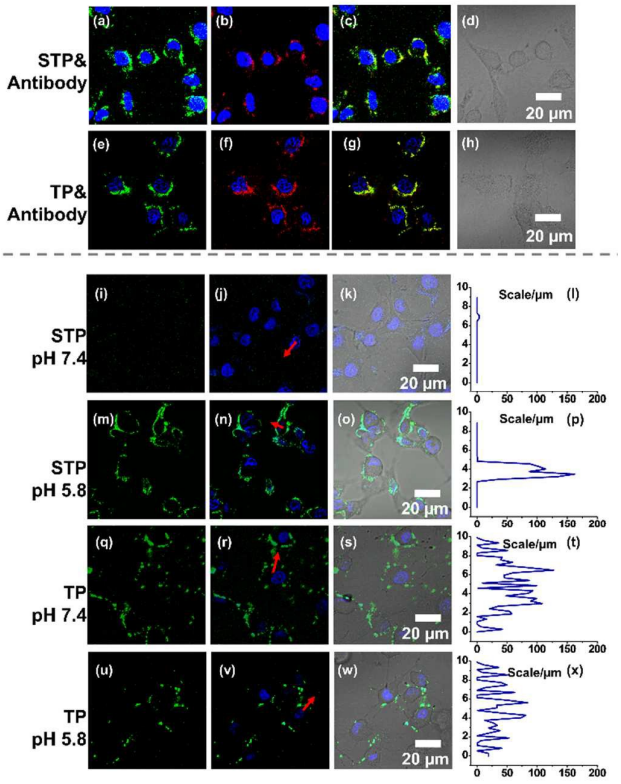

Figure 1. Specificity confirmation of the peptides toward VERFG2 in different conditions. FITC-STP interacted with HUVEC with PE-anti-human VEGFR2 (a-d). FITC-TP interacted with HUVEC with PE-anti-human VEGFR2 (e-h). The FITC labelled peptides targeted toward VEGFR2 were shown in green. PE-anti-human VEGFR2 were shown in red. Cell nucleus were stained with Hoechst 33342 (blue). FITC-STP binding to HUVEC at pH 7.4 (i-k) and at pH 5.8 (m-o). FITC-TP binding to HUVEC at pH 7.4 (q-s) and at pH 5.8 (u-w). The fluorescent intensity profiles along the red arrows through HUVEC membrane of STP and TP at pH 7.4 (l and t) and at pH 5.8 (p and x).

Next we estimated that peptide STP may show good penetrability for its specific binding behavior in acidic environment. Therefore, we carried out the confocal fluorescence imaging assay to monitor the peptide-cell interaction along with the time. Herein, FITC-labeled peptides were incubated with the cells in 0, 5, 10, 15, 20 min at pH 5.8. As shown in Figure 2a-e, FITC-STP showed the potential to slowly release into the cytoplasm along with the time and after 15 min FTIC-STP started to release into the cytoplasm and at 20 min the phenomenon was more obvious. For comparison, FITC-TP only showed affinity on the membranes at 20 min (Figure 2f-j). We supposed that the acidic microenvironment caused the protonation of amino acids and promoted FITC-STP binding to the VEGFR2 and penetrating into the cells. To further confirm the phenomenon of FITC-STP released into cytoplasm at 20 min, Z-stack Image was carried out (Figure S12). The dynamic cell image experiments further confirmed that FITC-STP had both pH dependent and targeting properties.

We tried to explain the penetrability phenomenon of STP in acidic environment. The sequence of STP was SKDEEWHKNNFPLSP, there was a unique segment "DEE" in the N terminal. It was reported that protonation of the Asp and Glu residues were associated with the conformation change from a random coil to an α-helix, which was one of the most important factors to form a penetrating peptide.[18] Therefore, Conformational dynamics simulations were performed first using the GROMACS software.[19] The potential energy of STP was calculated as -5.16×10^5 and the one of TP was calculated as -1.90×10^5 in acidic condition. It indicated that STP showed more stable structure in acidic environment. To further determine the real structure of the peptides, circular dichroism (CD) experiments were performed. As shown in Figure 3, STP had a signature CD spectral profile that consisted of a maximum at 190 nm, a minimum close to 208 nm and a second minimum at 222 nm at pH 5.8, which corresponding to the characteristic α-helix.[20, 21] While at pH 7.4, STP didn't show the characteristic signals. For comparison, the TP peptide almost showed none secondary structures. There were almost no difference at pH 5.8 and pH 7.4. The results of theoretical simulation were consistent with the experimental results, further confirmed our peptide STP had an α-helix structure at acidic condition.

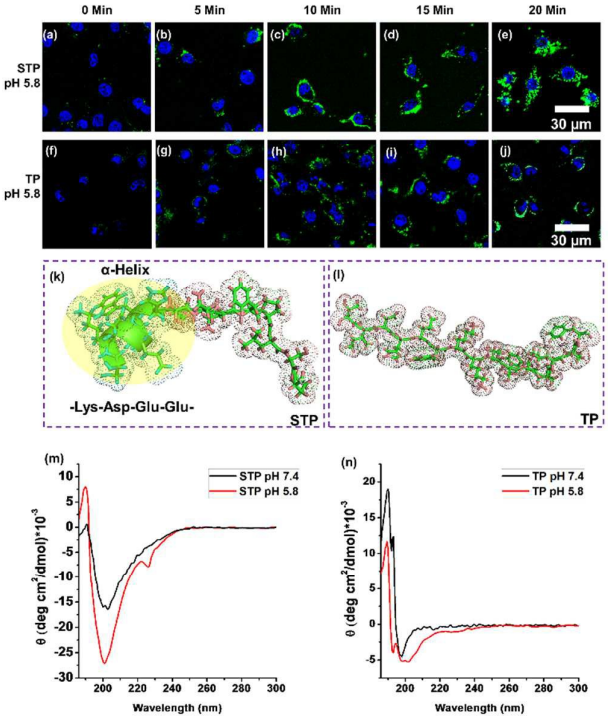

Figure 2. Confocal images of the FITC-labelled peptides binding to HUVEC cells at pH 5.8 with 0, 5, 10, 15, 20 min, respectively. FITC-STP binding to HUVEC cells (a-e). FITC-TP binding to HUVEC cells (f-j). The structure simulation (k, l) and circular dichroism spectra (m, n) of peptides at pH 5.8 (red) and pH 7.4 (black).

Finally, we tested the tumor vasculature-targeting efficacy *in vivo* and *ex vivo.* Here, the peptides STP and TP were labeled with carboxyl CdSe QDs. Human colonic adenocarcinoma cells HT-29 were reported expressing VEGFR2 proteins on its tumor blood vessel.[22] The peptides functionalized QDs were then injected into HT-29 xenograft mice and monitored by the small animal imaging system. The mice treated with only QDs was used as a negative

control. Figure 3a showed the real-time biodistribution and tumor accumulation of STP-QDs, TP-QDs and QDs at 2 h, 4 h, 8 h, 12 h and 24 h post-injection. The tumor fluorescence in the STP-QDs treated mice was notably higher than that in the TP-QDs treated ones at 8 h to 24 h post-injection. In addition, we observed almost no fluorescence localization in the corresponding regions of mouse that had received QDs. The *ex vivo* fluorescence images of excised tumors further confirmed that the high accumulation of the STP-QDs (Figure 3b and c). We supposed it owing to the high stability in the tumor acidic environment for its formation of an α-helix structure. To further trace the location of the Dye-labeled peptides and evaluation of the vasculature-targeting efficacy, the histological examination of the paraffin section of HT-29 tumor tissues was carried out. We also labeled the tumor vessels with fluorescence-tagged antibodies against the endothelial marker CD31, a vessel indicator. As depicted in the Figure 3d, STP-Cy5 treated tumors had obvious high fluorescence signals than that TP-Cy5 treated, which provided us directly insights into the evaluation of the vasculature-targeting efficacy in TME. These results confirmed that the Cy5-labeled STP showed a higher tumor vasculature-targeting efficiency, which was consistent with the *in vivo* imaging data.

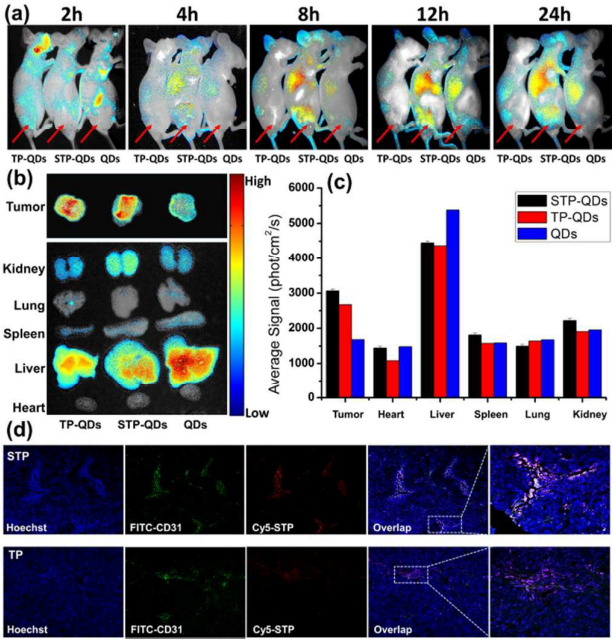

Figure 3. *In vivo* and *ex vivo* imaging of tumor-bearing mice using peptide probes.(a) *In vivo* optical imaging of nude mice bearing HT-29 tumors The tumors were visually indicated by the red arrows. (b) *Ex vivo* images of tumors and other tissues after 24 h post treatment. (c) Average fluorescence signals of major organs after 24 h post tail-vein injection. Values were expressed as means ±S.D. (N=3). (d) Paraffin section of tumor tissues that were removed after 24 h post treatment. Peptides were labeled with Cy5 (red). FITC-tagged CD31 antibody was used to label the tumor vessels (green).Cell nucleus were stained with Hoechst 33342 (blue).

The novel peptide probe STP towards VEGFR2 was successfully obtained with the distinctive pH triggered and targeting properties in the living cells and *in vivo* explant mice models. It also showed high vasculature-targeting efficacy. Last but not least, STP also showed the potential to slowly release into the cytoplasm along with the time. We expected this peptide-assisted probe could be used for endothelial vascular imaging, tumor positioning and diagnosis.

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Note

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