Electronic Supplementary Information (ESI)

Enzymatic activatable self-assembled peptide nanowire for targeted therapy and fluorescence imaging of tumors

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Experimental Section

**Reagents and materials.** Matrix metalloproteinase MMP2 proenzyme and p-aminophenylmercuric acetate were purchased from Merck Millipore (Darmstadt, Germany). MMP inhibitor N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH) was obtained from Sigma Aldrich (St. Louis, Mo, USA). CellTiter 96® AQueous One Solution Cell Proliferation Assay kit was from Promega (Madison, USA). HT-1080 cells and BT-20 cells were obtained from Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cell culture media were purchased from Thermo Scientific HyClone (MA, USA). Human red blood cells were provided by Xiangya Hospital (Changsha, China). Synthetic peptides including the four-domain probe, the control probe and the FAM-Q11 peptide were obtained from Shanghai GL Peptide Ltd (Shanghai, China). The sequences of these peptides are shown in Table S1. All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Concentrations of MMP2 used in *in vitro* assays were calculated directly using the amount of protein indicated by the provider (Merck Millipore) with no calibration. All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.25 MΩ.

**Preparation of the peptide nanowire probe.** The peptide nanowire probe was synthesized from self-assembly according to a procedure previously reported. Briefly, a mixture of Q11 (400 µM) and four-domain peptide (100 µM) in 1× phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) was firstly prepared. The self-assembly were performed by incubating the mixtures under static conditions overnight at room temperature. The nanowires were centrifuged at 12,000 g for 5 min and washed using 1× PBS twice. The sediments were re-dispersed in 1× PBS to a final concentration containing 100 µM four-domain peptide. Nanowires with other compositions of Q11 (400 µM) and four-domain peptide were synthesized using the same protocols. The concentrations of peptides in the nanowire solution were determined according to the absorbance readings at 490 nm and 280 nm. The molar absorption coefficients of the FAM-labeled Q11 peptide were determined to be 7300 M⁻¹ cm⁻¹ at 490 nm and 10280 M⁻¹ cm⁻¹ at 280 nm, and those for the four-domain peptide were 0 M⁻¹ cm⁻¹ at 490 nm and 6500 M⁻¹ cm⁻¹ at 280 nm, respectively, using the corresponding standard solutions (DMSO/H₂O 1:3 v/v). The absorbance of the nanowires was measured by re-dissolving the sediments obtained during centrifuge in 1:3 DMSO/H₂O (vol/vol), in which the nanowires were collapsed into free peptides.

For TEM characterization, the nanowires were 10-fold diluted and dropped on 300 mesh copper grids with carbon support films. Dried for 5 min, the nanowires were rinsed by double-distilled water for three times followed by staining using uranyl acetate (1.0 % w/v) solution for 5 min. The stained
samples were washed three times, dried followed by observation on a Hitachi H-7000 electron microscope (Tokyo, Japan) at an accelerating voltage of 200 kV.

CD measurements were performed on a MOS-500 Circular Dichroism Spectrometer (Le Pont-de-Clai, France). Solutions of peptide mixtures in water and nanowire solution were separately prepared for CD analysis. Each sample was analyzed three times and the spectra were averaged.

**In vitro assays for peptide nanowire probe.** The assay of MMP2 using the peptide nanowire probe was performed as follows: MMP2 (10 μL, 8.3 μM) was activated by incubating with 10 μL p-aminophenylmercuric acetate (2.5 mM) in 50 mM Tris buffer (pH 7.4) at 37 °C for 1 h.[S2] Afterward, we added 30 μL of peptide nanowire probe (100 μM four-domain peptide) solution and incubated the mixture in 50 mM Tris-HCl (pH 7.4) containing 200 mM NaCl, 10 mM CaCl$_2$, 1 mM ZnCl$_2$, 0.01% Brij at 37 °C for 2 h. Enzyme cleavage product was used for mass spectrometry (MS) analysis, and Zeta potential characterization as compared to the intact nanowire probe.

MS analysis was performed on a LTQ Orbitrap Velos Pro mass spectrometry (Thermo Fisher Scientific, Bremen, Germany). Nanowires before and after MMP2 cleavage were centrifuged at 12,000 g for 5 min, and the sediments were re-dissolved in 1:3 DMSO/H$_2$O (vol/vol) for MS analysis. The supernatant after the MMP2 reaction was also collected for the analysis of the cleaved products away from the nanowires.

Zeta potential measurements of the nanowires were performed at 25 °C on a Zetasizer Nano ZS90 Analyzer (Malvern Instruments, UK).

**Cell culture and fluorescence imaging.** HT-1080 and BT-20 cells were grown in DMEM medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100 U/mL gentamicin at 37 °C in a humidified atmosphere containing 5% CO$_2$. (**Note:** To avoid the loss of MMP2 in cell culture media during wash, the cell growth media were retained for subsequent experiments and the supplemented reagents were added in the growth media at a 100-fold higher concentration to avoid substantial dilution. Alternatively, a final concentration of MMP2 of 100 nM was added in the cell growth media when fresh DMEM medium was used.)

Cells were seeded in glass Petri dish and grown overnight. When the cells were ~80% confluent, the dishes were washed three times with 1× PBS. The cells were then incubated with 500 μL cell growth medium supplemented with the peptide nanowire probe (500 nM four-domain peptide) or the controlled peptide nanowire probe (500 nM) for 3 h followed by wash twice with fresh growth medium. The cells were then visualized by fluorescence imaging. In the experiment with MMP2 inhibitor NNGH, cells were incubated with 500 μL cell growth medium supplemented with the peptide nanowire probe (500 nM four-domain peptide) and in the presence of the inhibitor NNGH (500 μM, 5 μL) for 3 h.

All fluorescence images were acquired using an oil immersion objective (100×, NA 1.3) on a confocal laser scanning fluorescence microscope setup consisting of an Olympus IX81 inverted
microscope with an Olympus FV1000 confocal scanning system. Ar+ laser (488 nm) was used as excitation source, and a 505-560 nm bandpass filter was used for fluorescence detection.

Flow cytometric analysis of cells was performed on a FACScan cytometer (BD Biosciences, USA). HT-1080 cells (1×10^5 cells) were incubated separately with peptide nanowire probe (500 nM four-domain peptide), 500 nM control peptide nanowire probe, or peptide nanowire probe (500 nM four-domain peptide) plus 5 µM NNGH at 37 °C for 3 h. The cells was detached with 50 µL of 0.25% trypsin for 5 min and centrifuged for 5 min at 300 g followed by two washes with 500 µL 1× PBS and re-suspension in 1 mL PBS for flow cytometry assay.

**Cytotoxicity and hemolysis assays for peptide nanowire probe.** The cell viability was determined using a CellTiter 96® AQueous One Solution Cell Proliferation Assay kit. In the assay, HT-1080 cells and BT-20 cells were seeded in 96-well plates at 4×10^3 cells per well and incubated overnight before treatments. Growth medium (50 µL per well) containing peptide nanowire probe or free peptide probe of varying concentrations was added in cell-plated wells. Then, 20 µL CellTiter Reagent diluted with 50 µL of growth medium was added per well and incubated with the cells at 37 °C for 4 h followed by absorbance measurements at 492 nm using a Thermo Scientific Multiskan Microplate Reader (Thermo Fisher, USA).

Because there is no expression of MMP2 from human red blood cells (RBCs), the hemolysis assay was performed using the MMP-cleaved products for the peptide nanowire probe and the free peptide probe as compared to the intact probes. This assay gave direct evidences for the relative toxicity of the peptide nanowire probe and the free peptide probe. The MMP-cleaved products for the peptide nanowire probe and the free peptide probe were prepared by incubating the probes (100 µM) with 500 nM MMP2. In the hemolysis assay, RBCs (1.0 × 10^8 cells/mL in 1× PBS) was incubated with the peptide nanowire probe, the free peptide probe, and their MMP-cleaved products of varying concentrations in 1× PBS at 37 °C for 4 h. The absorbance of the supernatants from each group of RBCs was measured at 540 nm using a Thermo Scientific Multiskan Microplate Reader (Thermo Fisher, USA). RBCs treated with 1% Triton were used as a positive control, and the release rate of hemoglobin for this group was set at 100%.

**In vivo antitumor studies.** Female BALB/C nude mice (18 g, aged 4-5 weeks) were purchased from Hunan SJA Laboratory Animal Co. Ltd. (Hunan, China). All animal studies were performed in compliance with protocols that had been approved by the Hunan Provincial Animal Care and Use Committee and the experimental guidelines of the Animal Experimentation Ethics Committee of Hunan University. BALB/C mice were implanted subcutaneously with 4.6×10^6 HT-1080 cells in the right flank. When the tumor volume reached 100 mm³, the animals were randomly divided into two groups (n=5 per group), with each group having approximately equal starting average tumor size (100 mm³). The animals each received a single intratumoral injection of 100 mg/kg (3.9 µmol/kg of four domain peptide)
of the nanowire probe or the controlled nanowire probe followed an additional one-week treatment. The tumor volumes were measured at given time points for all animals. The day of the intratumoral injection was defined as day 0. The tumor size of each animal was measured using a caliper on the 3rd, 5th, 7th, 9th, 11th, 13th and 15th days. The tumor volumes were calculated according to the following formulae
\[ V = \frac{L \times W^2}{2}. \]

References:
**Table S1.** Sequences of synthesized peptides.\(^a\)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (N’ to C’)</th>
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<tbody>
<tr>
<td>four-domain peptide probe</td>
<td>EEE EEE EE PLG LAG IGA VLK VLT TGL PAL ISW IKR KRQ Q SGS GSG SG QQK FQF QFE QQ</td>
</tr>
<tr>
<td>Control peptide probe</td>
<td>EEE EEE EE GGP ALL IGA VLK VLT TGL PAL ISW IKR KRQ Q SGS GSG SG QQK FQF QFE QQ</td>
</tr>
<tr>
<td>FAM-Q11</td>
<td>FAM-QQK FQF QFE QQ</td>
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\(^a\) The four-domain peptide includes the polyanionic domain (black), the substrate peptide (blue), melittin (red), a flexible linker (brown) and Q11 (green). Control peptide has a scrambled substrate sequence (purple), which cannot be cleaved by MMP2.
Fig. S1. CD spectra for peptide self-assembly. Blue line was the spectra for peptide without self-assembly (in salt-free solution), and black line was the spectra for peptide assemblies. (a) 400 µM four-domain peptide, (b) 400 µM four-domain peptide plus 400 µM Q11, (c) 200 µM four-domain peptide plus 400 µM Q11, (d) 100 µM four-domain peptide plus 400 µM Q11.

It is observed that only the mixed assembly of 100 µM four-domain peptide with 400 µM Q11 give an appreciable positive peak at 206 nm and a negative peak at 229 nm in the CD spectrum, typical for self-assembled nanowires of Q11 peptide. There is no positive peak appearing for mixed assembly of 400 µM Q11 with 200 µM or 400 µM four-domain peptide, indicating lower concentration of Q11 in the mixed assembly process is incapable of self-assembling into Q11-based nanowires. There is no significant change in the CD spectra for self-assembly of 400 µM four-domain peptide, implying direct self-assembly of the four-domain peptide does not yield substantial amount of nanowires.
Fig. S2. Zeta potential analysis for nanowires before (a) and after (b) cleavage by MMP2.
**Fig. S3.** MS analysis for the nanowire probe. (a) Peptide nanowire probe before reaction with MMP2, (b) Sediment product after reaction of peptide nanowire probe with MMP2, (c) Supernatant product after reaction of peptide nanowire probe with MMP2. Major peaks in (a) indicate the parent ion for the four-domain peptide (obs. 6375.25 Da), while major peaks in (b) and (c) reveal the parent ions for two cleaved fragments, LAG-melittin-Q11 (obs. 5074.75 Da) and EEEEEEE-PLG (obs. 1317.48 Da).
Fig. S4. Confocal microscopy images with more cells. (a) Nanowire probe incubated with BT-20 cells for 3 h; (b) Nanowire probe incubated with HT-1080 cells for 3 h; (c) Nanowire probe incubated with HT-1080 cells in the presence of 5 µM inhibitor NNGH for 3 h; (d) Control nanowire probe incubated with HT-1080 cells for 3 h. The concentration of the four-domain peptide was 500 nM in the experiments.
Fig. S5. Flow cytometric assay of HT-1080 cells. Cells only (blue), cells incubated with control nanowire probe for 3 h (yellow), cells incubated with nanowire probe in the presence of 5 μM inhibitor NNGH for 3 h (red), and cells incubated with nanowire probe (green). The concentration of the four-domain peptide was 500 nM in the experiments.
Fig. S6. Hemolysis assay. (a) Photograph for hemolysis assay, (b) Hemoglobin release assay. RBCs incubated with free peptide probe (black) or nanowire probe (blue) without MMP2 cleavage, and RBCs incubated with cleaved products for free peptide probe (red) or nanowire probe (green).