*Electronic Supplementary Information for*

# Rational development of molecularly imprinted nanoparticles for blocking PD-1/PD-L1 axis

Zikuan Gu[#], Shuxin Xu[#], Zhanchen Guo and Zhen Liu*

State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210023, PR China

* To whom correspondence should be addressed. E-mail: zhenliu@nju.edu.cn

## Experimental Procedures

### Reagents and Materials

Cyclohexane, 1-hexanol, acetonitrile and acetone were purchased from Aladdin (Shanghai, China). Triton X-100, phorbol-12-myristate-13-acetate (PMA), phytohemagglutinin (PHA), cytochrome C (Cyt C), ribonuclease B (RNase B), horseradish peroxidase (HRP) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Interferon-γ (IFN-γ) was purchased from R&D system (California, USA). 3-Aminopropyltriethoxysilane (APTES), tetraethyl orthosilicate (TEOS), isobutyltriethoxysilane (IBTES), 3-ureidopropyltriethoxysilane (UPTES) and benzyltriethoxysilane (BnTES) were purchased from J&K scientific (Beijing, China). Ammonium hydroxide, acetic acid and ethanol were purchased from Nanjing Chemical Reagent (Nanjing, China). PD-1 epitope peptide (FLDSPDRPWN, purity 98%), hydrophobic C13 fatty acid chain modified PD-1 epitope peptide (FLDSPDRPWNK-C13, purity 98%), Fluorescein isothiocyanate isomer (FITC)-modified PD-1 epitope (FLDSPDRPWNK-FITC, purity 98%) was provided by Top-Peptide (Shanghai, China). Jurkat T cells and 786-O cells were purchased from Nanjing Liao Biotechnology (Nanjing, China). Anti-PD-L1 antibody (Alexa Fluor®488) was purchased from Abcam (London, UK.). PE-labeled human PD-1. Fc tag. His tag, biotinylated PD-1 and PD-L1 Inhibitor screening ELISA Assay pair were purchased from ACROBiosystems (Beijing, China). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (New York, USA). Nivolumab (MB2726) was purchased from Meilunbio (Dalian, China). Phosphate-buffered saline solutions for cell culture (1xPBS), Roswell Park Memorial institute 1640 medium (RPMI-1640, containing 2.0mg/mL D-glucose, 0.3 mg/mL glutamine, 2.0 mg/mL $NaHCO_3$, 80 U/mL penicillin, and 0.08 mg/mL streptomycin), dimethyl sulfoxide (DMSO), parenzyme cell digestion solution (containing 0.25% trypsin and 0.02% EDTA), and 3-(4.5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) were purchased from Keygen Biotech (Nanjing, China). Human interleukin-2 (IL-2) ELISA kit was purchased from Meimian (Jiangsu, China).

**Instruments**

Transmission electron microscopic (TEM) characterization was performed on a JEOL JEM-2100 TEM instrument (Tokyo, Japan). UV-Vis absorption was recorded on a NanoDrop spectrometer from ThermoFisher Scientific (Waltham, USA). Fluorescence characterization and MTT assay were performed on a SynergyMX from Bio Tek (Winooski, USA). Flow cytometry was performed on a flow cytometer (Beckman Coulter FC, USA). The BLI binding assay was performed on an Octet Red 96 instrument (ForteBio, CA, USA). Diameter distribution was characterized on a BI-200SM (Holtsville, NY, USA)

**Molecular dynamic simulation**

The structure of PD-1-nivolumab Fab complex was retrieved from the PDB data bank (ID:5WT9), visualized and prepared by the PyMOL software. The hydrogen bond interactions between PD-1 and nivolumab Fab was analyzed by PyMOL software.

**Reverse microemulsion formation**

1.77 g of Triton X-100, 1.6 mL of 1-Hexanol, 6.5 mL of cyclohexane, 480 μL of deionized water and 100 μL of ammonium hydroxide were added into a 25-mL eggplant-shaped flask with Teflon-coated magneton. Then the mixture was stirred at 700 rpm under 25 °C for 30 min to form the reverse microemulsion.

**Epitope imprinting**

1 mg of C13 modified PD-1 epitope peptide (FLDSPDRPWNK-C13) was added into the aboved microemulsion and stirred for 30 min. During this procedure, the following steps were performed: adding 100 μL of APTES, UPTES, IBTES and BnTES with different ratios into 1 mL of cyclohexane to prepare a monomer solution, which is designed as L1; adding 100 μL of TEOS into 1 mL of cyclohexane and the solution is named as L2; mixing L1 and L2 at the ratio of 3:7 to form 1 mL of the solution which was named as L3. Then, L3 was added into the microemulsion prepared above. The mixture was stirred at 700 rpm at 25 °C for 24 h. The prepared molecularly imprinted

polymers nanoparticle was released from the microemulsion by adding acetone, followed by centrifugation at 4000 rpm for 30 min to separate from the mixed solution. The obtained nanoparticle was washed with ethanol and water for three times, respectively.

**Template removal**

The obtained nanparticle was dispersed into 10 mL of eluent (ACN: $H_2O$: HAc= 50: 49: 1(v/v)) and shaken for 1 h at room temperature. After removing the C13-grafted epitope template, the nanoparticle (termed as anti-PD-1 nanoMIP) was collected by centrifugation at 4000 rpm for 30 min. The obtained anti-PD-1 nanoMIP was washed with water and ethanol for three times, respectively and then frozen dried in a vacuum overnight. The product was stored at 4 °C. The corresponding non-imprinted nanoparticles (nanoNIP) were prepared with the same process except for the use of template molecule.

**Optimization of monomers ratio.**

The monomer ratio used for the preparation of anti-PD-1 nanoMIP was optimized in terms of the obtained imprinting factor (IF). The chemical properties of template peptide sequence were firstly analyzed (Figure S1). According to our previous study,[1] amino acids are categorized into five classes. Based on this principle, the peptide sequence was composed of two acid amino acid (pink), one basic amino acid (blue), two aromatic amino acid (brown), three hydrophobic amino acid (green), and two other amino acid (black). (FigureS1) According to the chemical properties, APTES was chosen to provide electrostatic force with acid amino acid, and hydrogen bond force with other amino acid; UPTES was chosen to provide hydrogen bond force with basic and other amino acid; BnTES was chosen to provide $\pi$-$\pi$ stacking interaction with aromatic amino acid; IBTES was used to provide hydrophobic force with hydrophobic amino acid. TEOS mainly served as a crosslinker to form a silica skeleton and helped to reduce the non-specific adsorption. Then, the candidate monomer ratios were designed and optimized by experimental results. Briefly, anti-PD-1 nanoMIP and

nanoNIP prepared with different monomer ratio (APTES, UPTES, IBTES, BnTES) were dispersed into 200 μL of phosphate buffer (10 mM, pH = 7.4) containing 0.5 mg/mL of PD-1 epitope peptide (FLDSPDRPWN). After incubation for 1 h, the anti-PD-1 nanoMIP and nanoNIP were collected via centrifugation and rinsed with phosphate buffer (10 mM, pH = 7.4) for three times, The obtained anti-PD-1 nanoMIP and nanoNIP were eluted with 20 μL of eluent (ACN: $H_2O$: HAc = 50: 49: 1) with shaking for 30 min. The amount of PD-1 epitope peptide captured by anti-PD-1 nanoMIP or nanoNIP were determined by measuring the UV absorbance of eluent at 214 nm. Three parallel samples were measured for each group. The IF value was calculated according to the ratio of the amount of epitope peptide captured by anti-PD-1 nanoMIP over that by nanoNIP.

**Selectivity test of anti-PD-1 nanoMIP at peptide level**

The selectivity of anti-PD-1 nanoMIP at peptide level was evaluated using PD-1 epitope peptide (FLDSPDRPWN) and other interfering peptides (IVGGWECEK; GDVEKGKKI; APMAEGGGQNHH; NYKELGFQQ) as the test peptide. Briefly, the standard solutions (0.5 mg/mL) of each peptide were prepared with phosphate buffer (10 mM, pH = 7.4). Then 2 mg of anti-PD-1 nanoMIP was dispersed into 200 μL of peptide solution. The dispersion was shaken on a rotator at room temperature for 30 min. After shaking, the nanoparticles were collected by centrifugation at 4000 rpm for 30 min and then washed with phosphate buffer (10 mM, pH 7.4) for three times. Furthermore, the nanoparticles were re-suspended and eluted in 20 μL of eluent (ACN: $H_2O$: HAc = 50: 49: 1) at room temperature for 30 min on a rotator. The amount of peptide captured by the anti-PD-1 nanoMIP was determined by measuring the UV absorbance of the supernatant at 214 nm.

**Selectivity test of anti-PD-1 nanoMIP at protein level**

The selectivity of anti-PD-1 nanoMIP at protein level was evaluated using the Human PD-1 protein and other interfering proteins (Cyt C, RNase B; HRP; BSA) as the test protein. Briefly, the standard solutions (200 μg/mL) of each protein were prepared with

phosphate buffer (10 mM, pH 7.4), and then 2 mg of anti-PD-1 nanoMIP was dispersed in 200 μL of protein solution. The dispersion was shaken on a rotator at room temperature for 30 min. After shaking, the nanoparticles were collected by centrifugation at 4000 rpm for 30 min and then washed with phosphate buffer (10 mM, pH 7.4) for three times. After that, the nanoparticles were re-suspended and eluted in 20 μL of eluent (ACN: $H_2O$: HAc = 50: 49: 1) at room temperature for 30 min on a rotator. The amount of the protein captured by the anti-PD-1 nanoMIP was determined by measuring the UV absorbance of the supernatant at 260 nm.

**Calibration curves of FITC-PD-1 epitope**

A series of standard solution of FITC-PD-1 epitope peptide (1, 3.16, 10, 31.6, 100, 316, 1000, 3160, 10000 ng/mL) were prepared with phosphate buffer (10 mM, pH 7.4) and placed in 96-well plate. The emission fluorescence signals obtained by the Synergy MX (excitation wavelength: 488 nm; emission wavelength: 520 nm) were used for fitting the calibration curve of FITC-PD-1 epitope peptide.

**Adsorption isotherm of anti-PD-1 nanoMIP to PD-1 epitope**

A series of standard solution of FITC-PD-1 epitope peptide (1, 3.16, 10, 31.6, 100, 316, 1000, 3160, 10000 ng/mL) were prepared with phosphate buffer (10 mM, pH 7.4), and then 2 mg of anti-PD-1 nanoMIP was dispersed into the standard solutions (200 μL). The dispersions were shaken on a rotator at room temperature for 30 min. After shaking, the anti-PD-1 nanoMIP was separated via centrifugation and the supernatants were collected. Then the supernatants were added into the 96-well plate. The emission fluorescence signals were obtained by the Synergy MX (excitation wavelength: 488 nm; emission wavelength: 520 nm). At last, the amount of the FITC-PD-1 epitope peptide captured by the anti-PD-1 nanoMIP was calculated by the fluorescence signal difference between the standard solution and the supernatant. The adsorption isotherm was established by plotting the data above. To estimate the binding affinity to FITC-PD-1 epitope peptide, the amount of FITC-PD-1 epitope peptide bound by the anti-PD-1 nanoMIP was plotted according to the Hill equation as given below:

$$\mathrm{Log}[(1-\theta)/\theta] = \mathrm{Log}K_d - n\mathrm{Log}\,[\text{Free Concentration}]$$

Where $\theta$, $n$ and $K_d$ are the ratio of adsorption capacity to the saturated adsorption capacity, Hill coefficient and the dissociation constant, respectively. By plotting Log[ $(1-\theta)/\theta$] versus Log[Free Concentration], $K_d$ and $n$ could be calculated.

**Dissociation constant of anti-PD-1 nanoMIP to PD-1 protein**

The dissociation constant of anti-PD-1 nanoMIP to PD-1 protein was determined using a real-time, label free, bio-layer interferometry (BLI) assay. The entire experiment was conducted at 25 °C. The test plate was shaking at the speed of 1000 rpm. The dispersion anti-PD-1 nanoMIP (200 μg/mL) was added into the black 96-well plate and loaded on Aminopropylsilane (APS) probe for 5 min. Then the APS probe was immersed in phosphate buffer (10 mM, pH 7.4) with 0.5 % of Tween-20 to clad the action sites without modifying anti-PD-1 nanoMIP. A series of standard solution of PD-1 protein (1.25, 2.5, 5, 10, 20 μg/mL) were prepared with phosphate buffer (10 mM, pH 7.4). Phosphate buffer without PD-1 protein was used as the control group. Then, the APS probes modified with anti-PD-1 nanoMIP were associated with the series of standard solution of PD-1 protein in 96-well plate until saturation, followed by dissociation in the phosphate buffer (10 mM, pH 7.4) for 5 min in 96-well plate. Finally, BLI Label-Free Detection System was used to fit the dissociation constant during the process. The probe without PD-1 protein modification was used as the control group.

**ELISA analysis of anti-PD-1 nanoMIP blocking the PD-1/PD-L1 interaction**

Recombinant human PD-1 protein was coated on the ELISA 96-well plate at 4 °C overnight. Anti-PD-1 nanoMIP and nanoNIP were added into wells for PD-1 binding. After washing with phosphate buffer (10 mM, pH 7.4) for three times, biotinylated PD-L1 protein was added into the wells followed by shaking on the rotator for 1h. After washing with phosphate buffer (10 mM, pH 7.4), PD-L1 protein remained in the wells were detected by Biotin/Streptavidin-HRP system. Wells without anti-PD-1 nanoMIP and nanoNIP adding were used as the control group. Wells without any nanoparticles

and proteins were used as the background group. Absorbance was measured at a wavelength of 490 nm. The binding rate expressed as a percentage of the absorbance of test group (added with nanoparticles) over the control experiment (without any nanoparticles) (both were deducted by the background absorbance), which can be calculated by the following equation:

$$\text{Binding rate (\%)} = \frac{\text{Abs(test)} - \text{Abs(background)}}{\text{Abs(control)} - \text{Abs(background)}} \times 100\%$$

**Cell culture**

Human renal carcinoma cell line 786-O was used as cancer cell model with high PD-L1 expression. Human T lymphocyte cell line Jurkat was used as the PD-1-expressing immune cell model. The two cell lines were all cultured in RPMI 1640 media. The cell culture media culturing 786-O cells was supplemented with 10% (v/v) FBS. The cell culture media culturing the Jurkat T cells was supplemented with 15% (v/v) FBS. All cells were incubated under 5% $CO_2$ at 37°C.

**Activation of PD-L1 expression on the surface of 786-O cells**

The PD-L1 expression by 786-O cells was activated by IFN-γ. Briefly, 786-O cells were harvested and seeded in 6-well plates at concentration of $1\times10^5$ cells/well. After that, 1 μL of IFN-γ solution (0.2 mg/mL) was added into the 6-well plate to simulate PD-L1 expression. After incubation for 24 h, the cells were enzymatically hydrolyzed from the 6-well plate followed by incubating with the anti-PD-L1 protein (Alex®488) for 30 min. After washing for three times with phosphate buffer (10 mM, pH 7.4), flow cytometry assay was used to detect the expression of PD-L1 on cells surface.

**In vitro cytotoxicity of anti-PD-1 nanoMIP and nanoNIP**

Cell viability was tested by the MTT assay. Briefly, 786-O and Jurkat T cells were harvested and seeded in 96-well plate at concentration of 5000 cells/well. The cells were incubated with different concentration of anti-PD-1 nanoMIP and nanoNIP (12.5,

25, 50, 100, 200, 300, 400 μg/mL) at 37 °C for 24 h. The cells without nanoparticles were used as control group and wells without cells were used as background group. Then, 50 μL of MTT indicator dye (1 mg/mL in phosphate buffer) were added. After incubating for another 4 h at 37 °C in the dark, 100 μL of Formazan solution was added into each well. After incubating for 4 h at 37 °C, the optical density of the solution was monitored on the Synergy MX. Absorbance was measured at a wavelength of 550 nm. The cell viability was expressed as a percentage of the absorbance of test cells (added with nanoparticles) over the control experiment (without any nanoparticles) (both were deducted by the background absorbance), which can be calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Abs(test)} - \text{Abs(background)}}{\text{Abs(control)} - \text{Abs(background)}} \times 100\%$$

**Anti-PD-1 nanoMIP blocking the PD-1/PD-L1 interaction at cell level**

Briefly, 786-O cells were harvested and seeded in 6-well plates at concentration of $1\times10^5$ cells/well. After that, 1 μL of IFN-γ (0.2 mg/mL) was added into the 6-well plate to simulate PD-L1 expression. 100 μL of PE-PD-1 protein (10 μg/mL) was mixed with 2 mg of anti-PD-1 nanoMIP in a 250 μL microcentrifuge tubes, which were shaken on the rotator for 30 min at 4 °C. Then the supernatant was collected by centrifugation. Finally, the supernatant was added into the 786-O cells and shaken on the rotator for 30 min at 4 °C followed by washing for three times. The fluorescence signal of PE-PD-1 protein binding on the cell surface was analyzed by flow cytometry.

**Assessment of T cell cytokine production**

Cytokine production of Jurkat T cells was investigated in a cancer-immune cell co-culture system by assessing the amount of IL-2 secreted by T cells using a human IL-2 ELISA kit. Briefly, 786-O cells were harvested and seeded in 96-well plates at concentration of 5000 cells/well for 24 h. Jurkat T cells were cultured in 25 $cm^2$ flask. 786-O cells and T cells were pre-treated with IFN-γ (0.05 μL, 0.2 mg/mL) and PHA (20

μL, 0.5 mg/mL)/ PMA (5 μL, 0.1 mg/mL) for 30 h in order to induce PD-L1 and PD-1 expression, respectively. Jurkat T cells were then treated with Nivolumab, anti-PD-1 nanoMIP or nanoNIP for 30 min followed by being co-cultured with the 786-O cells for 24 h. The ration of 786-O cells and Jurkat T cells was set at 1:4. Cell culture supernatant was then collected after 24 h incubation and assessed for IL-2. Cells without any materials added were used as the control group.

**Chemoresistance depending on the DOX concentration**

MTT assay was utilized to quantitatively examine the chemoresistance of 786-O and Jurkat T cells co-cultured model to DOX. 786-O cells were seeded in 96-well plates at concentration of 5000 cells/well for 24 h. Jurkat T cells were cultured in 25 $cm^2$ flask. 786-O cells and T cells were pre-treated with IFN-γ and PHA/PMA, respectively. Then, 786-O cells and Jurkat T cells were incubated at a ratio of 1:4, followed by adding a series of standard solution of DOX (1.25, 5, 20, 80 μM). After 4 h, 786-O cells were washed with the phosphate buffer (10 mM, pH 7.4) for three times, and the cell viability was determined by the MTT assay. The cells without DOX were used as control. And wells without cells were used as background group.

**Chemoresistance assay**

The chemoresistance assay was performed by measuring the synergistic cytotoxicity effect of immune checkpoint blockade and DOX. 786-O cells were seeded on 96-well plates at the concentration of 5000 cells/well and Jurkat T cells were cultured in 25 $cm^2$ flask. 786-O cells and T cells were pre-treated with IFN-γ and PHA/PMA, respectively. Nivolumab (5 μg/mL), anti-PD-1 nanoMIP (200 μg/mL) and nanoNIP (200 μg/mL) were added into the Jurkat T cells. After 30 min, 786-O cells and Jurkat T cells were co-cultured at a ratio of 1:4 for 24 h, followed by adding DOX (5 or 20 μM) for 4h. Then, the cells were washed with the phosphate buffer (10 mM, pH 7.4) for three times, and the cell viability was determined by the MTT assay. The cells without DOX were used as control and wells without cells were used as background group. Absorbance was measured at a wavelength of 550 nm. The cell viability expressed as a percentage of

the absorbance of test cells over the control experiment (both were deducted by the background absorbance), which can be calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Abs(test)} - \text{Abs(background)}}{\text{Abs(control)} - \text{Abs(background)}} \times 100\%$$

**Data availability**

The PDB accession codes 5WT9 and the UniProt accession code Q15116 were used in this study. All other relevant data are available from the corresponding authors upon reasonable request.

**Reference:**

[1] Z. C. Guo, R. R. Xing, M. H. Zhao, Y. Li, H. F. Lu and Z. Liu, *Adv. Sci*. **2021**, 2101713.

**Supplementary Data**

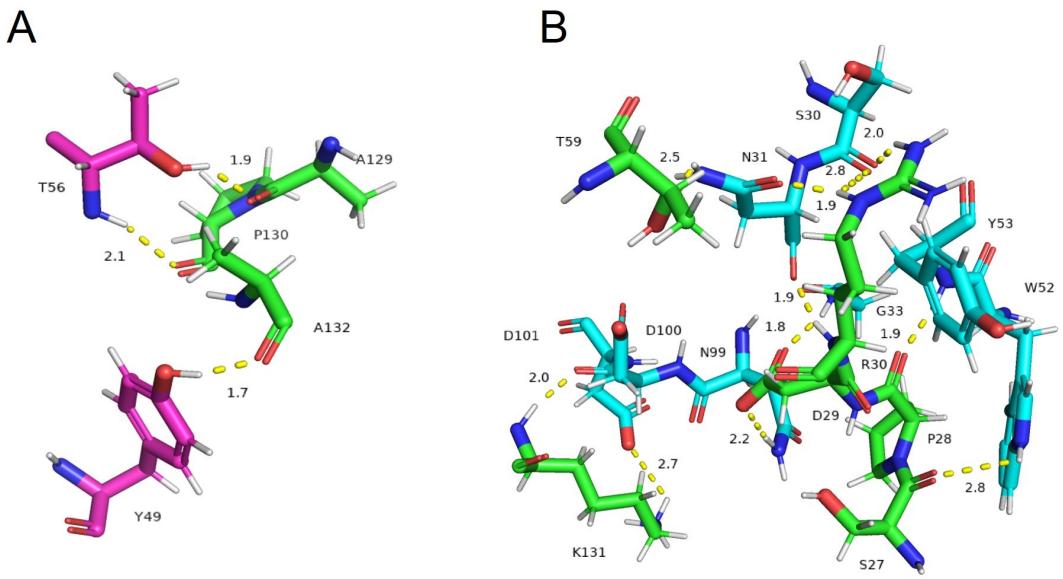



**Figure S1**. The atomic interaction details at the binding interface of PD-1-nivolumab Fab complex. Detailed interactions of PD-1 binding to the light chain (A) and heavy chain (b) of nivolumab. Residues involved in the hydrogen bond interaction are shown as sticks and labelled. Hydrogen bonds are shown as dashed yellow lines. PD-1: green; light chain of nivolumab: magenta; heavy chain of Nivolumab: light blue

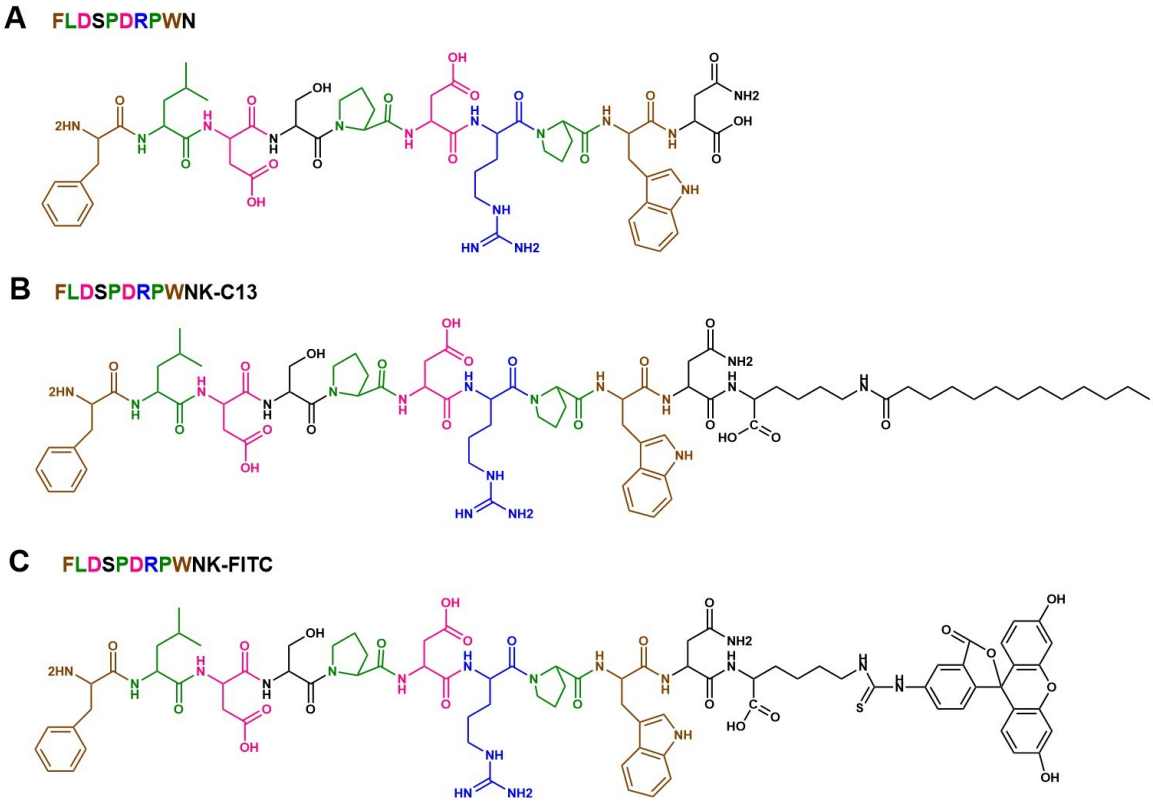
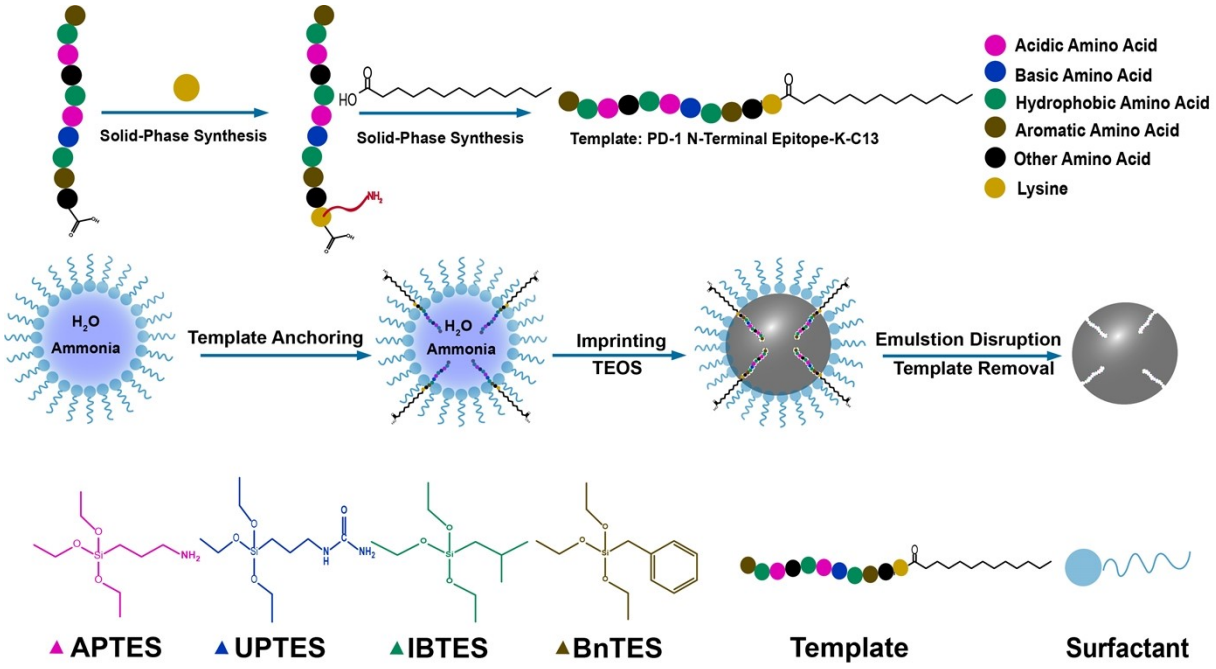



**Figure S2**. The amino sequence of A) PD-1 epitope peptide; B) hydrophobic C13 fatty acid chain modified PD-1 epitope peptide; C) FITC modified PD-1 epitope; (pink: acid amino acid; blue: basic amino acid; brown: aromatic amino acid; green: hydrophobic amino acid; black: other amino acid).




**Scheme S1**. Schematic illustration of the reverse microemulsion-confined epitope-oriented surface imprinting.

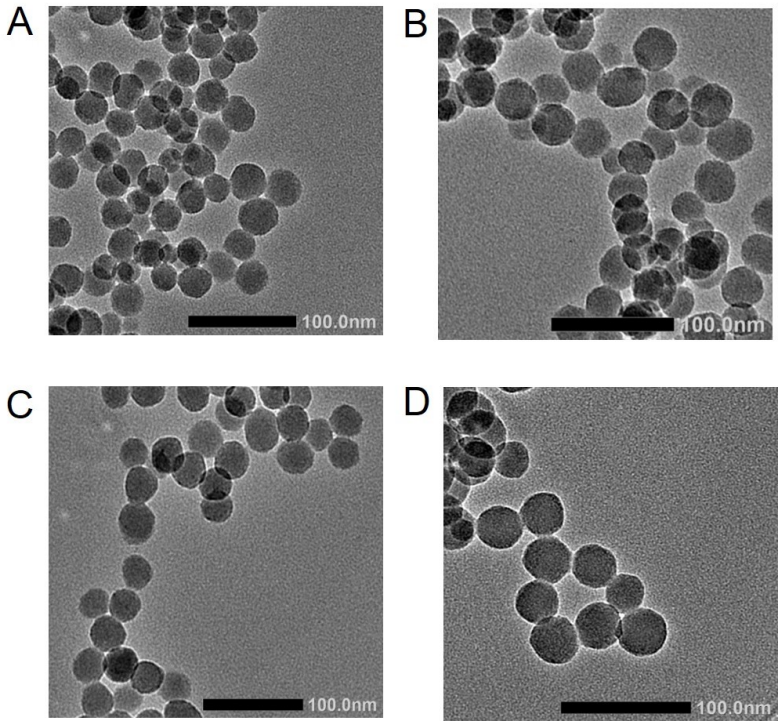



**Figure S3**. TEM images for the nanoparticles prepared by different proportions of the monomers. TEOS: APTES: UPTES: IBTES: BnTES = A) 70: 4.5: 4.5: 15: 6; B) 70: 6: 3: 15: 6; C) 70: 9: 3: 12: 6; D) 70: 15: 3: 6: 6.

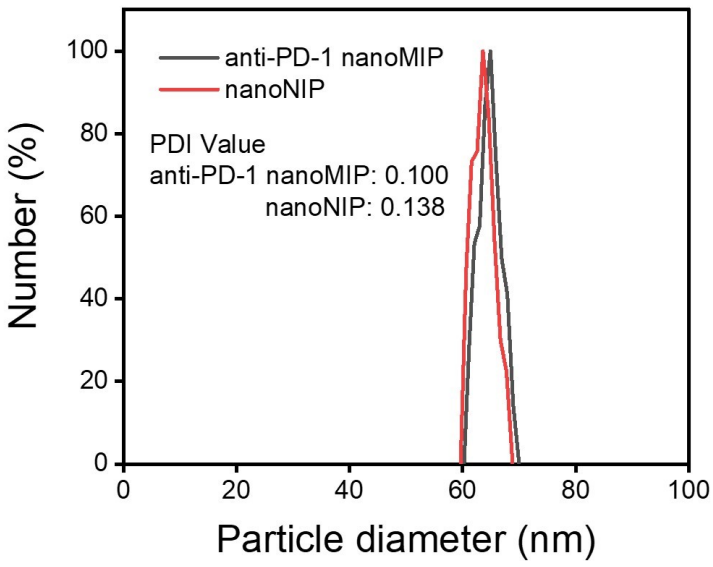


**Figure S4**. DLS characterization of anti-PD-1 nanoMIP and nanoNIP.

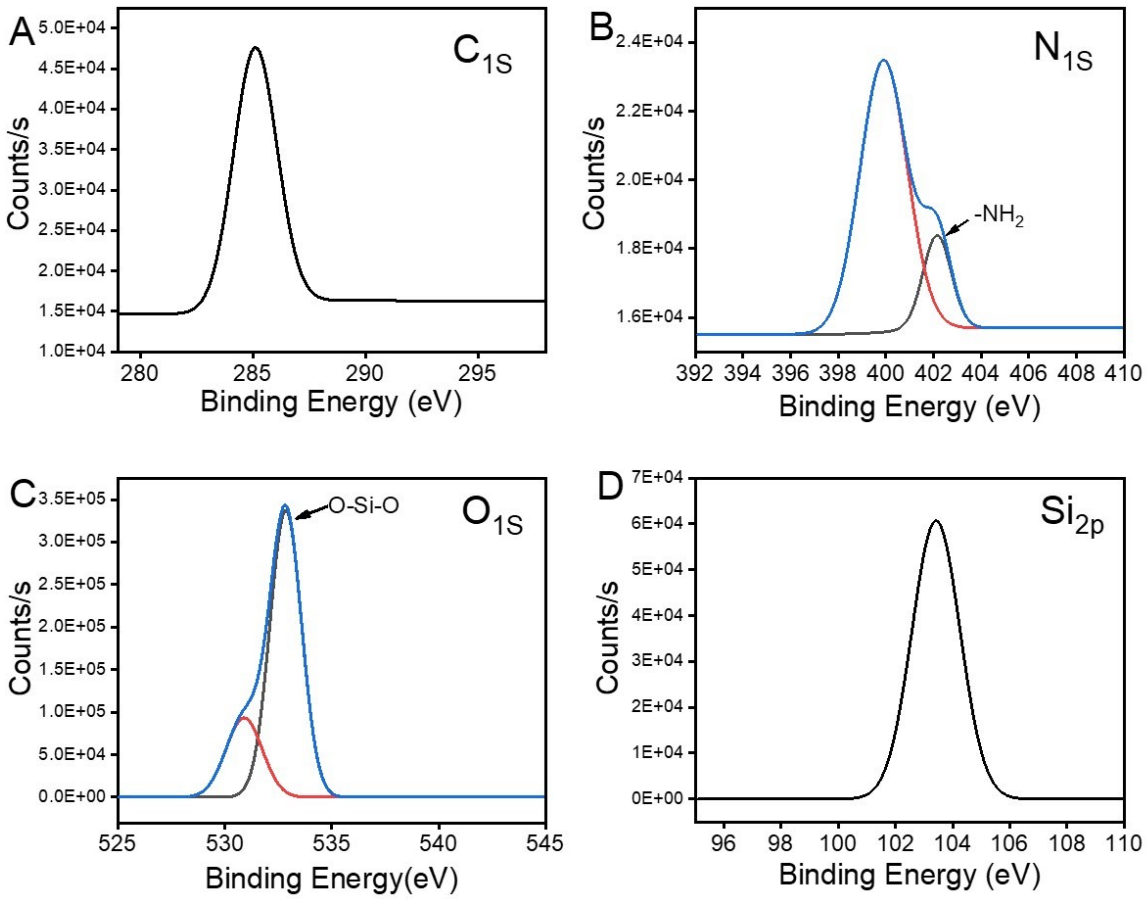



**Figure S5**. High-resolution XPS spectrum of A) $C_{1S}$; B) $N_{1S}$; C) $O_{1S}$; D) $Si_{2p}$.

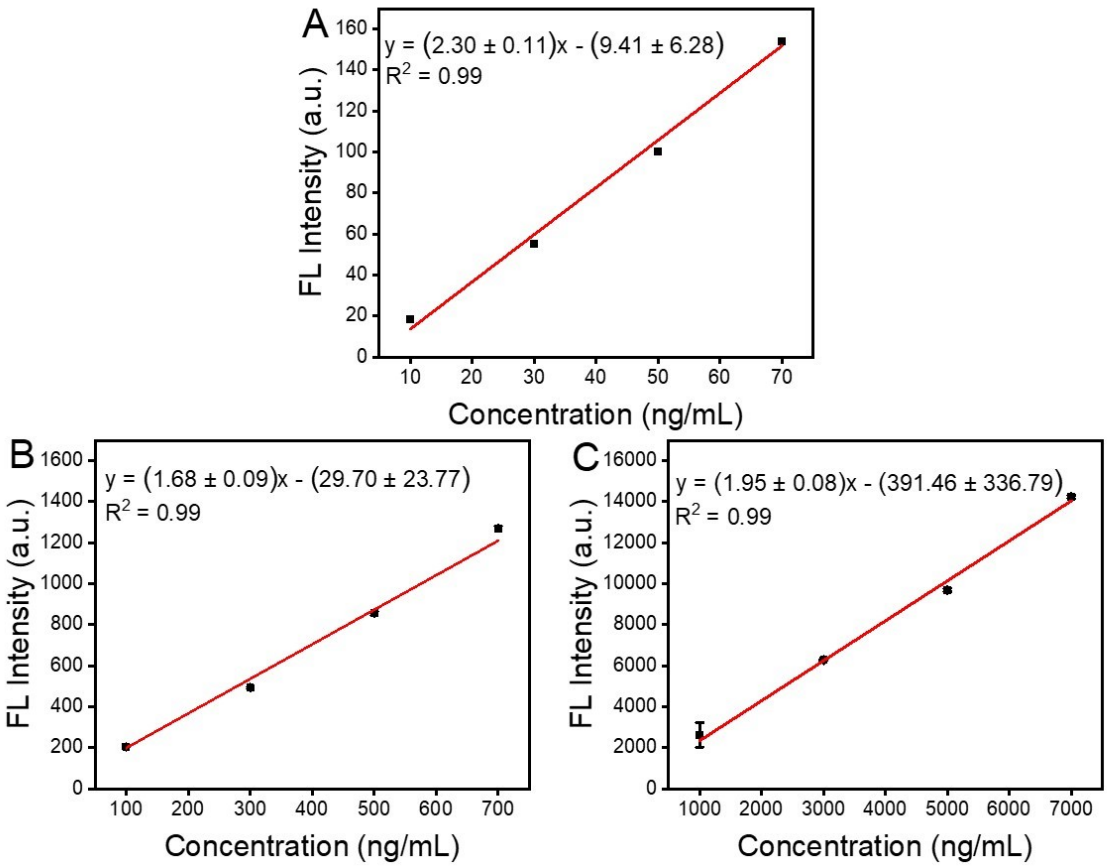



**Figure S6**. Calibration curve of FITC-PD-1 epitope (A: 1 - 70 ng; B: 100 - 700 ng; C: 1000 - 7000 ng). Data are presented as Mean ± SD (n = 3)

.

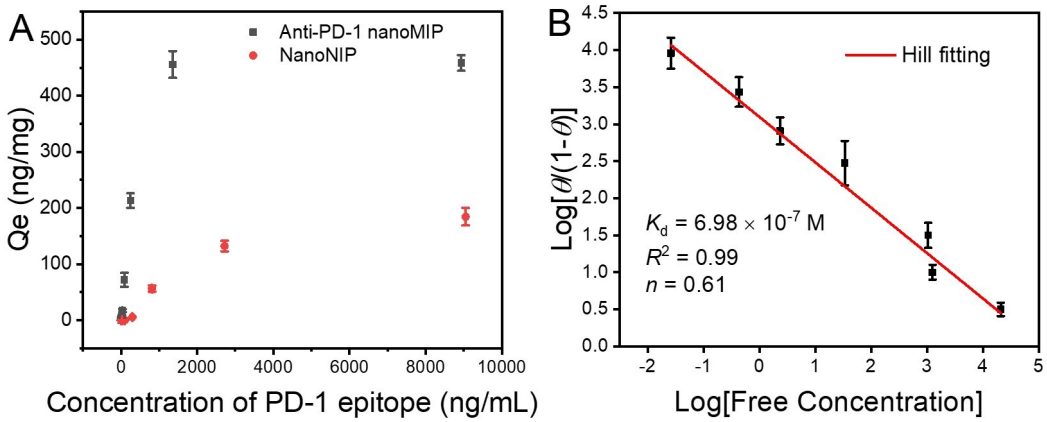



**Figure S7**. A) Adsorption isotherm of anti-PD-1 nanoMIP and nanoNIP to FITC-PD-1-epitope peptide. Qe: the amount of the target compound bond by anti-PD-1 nanoMIP and nanoNIP at equilibrium; B) Binding isotherms of nanoNIP to PD-1 epitope peptide (Hill fitting: Log[$\theta$/(1-$\theta$)] = Log$K_d$ – $n$Log[Free Concentration]); Data are presented as Mean ± SD (n = 3)

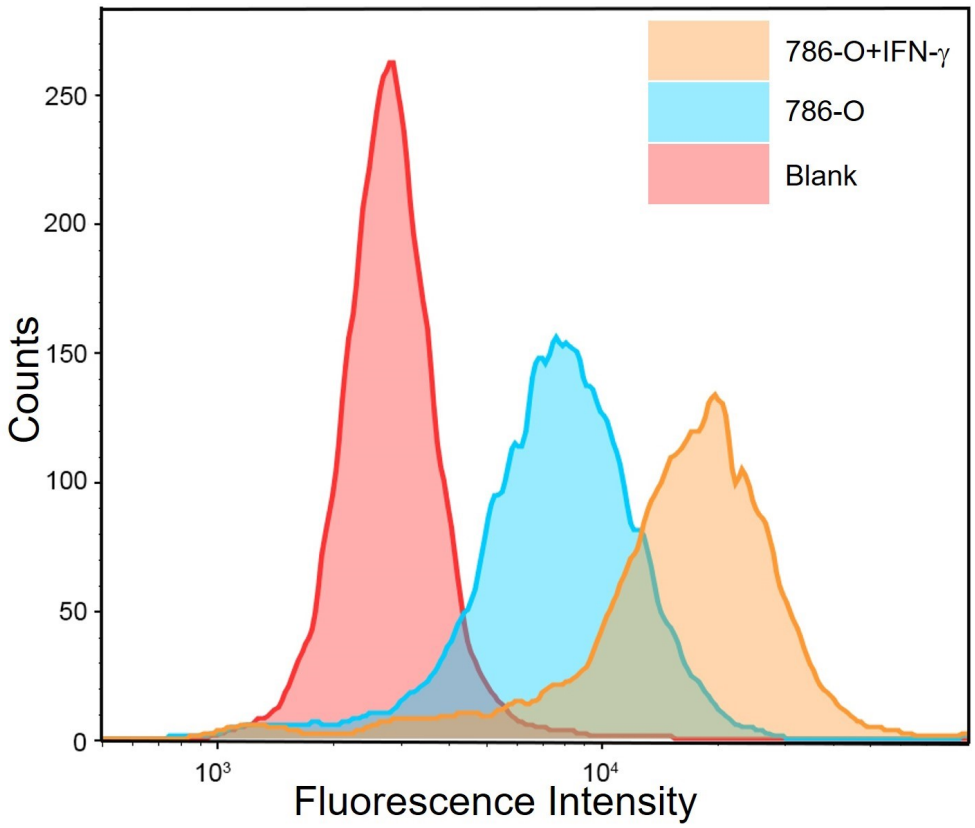



**Figure S8.** Flow cytometry analysis for PD-L1 expression on the surface of 786-O cells induced by IFN-γ.

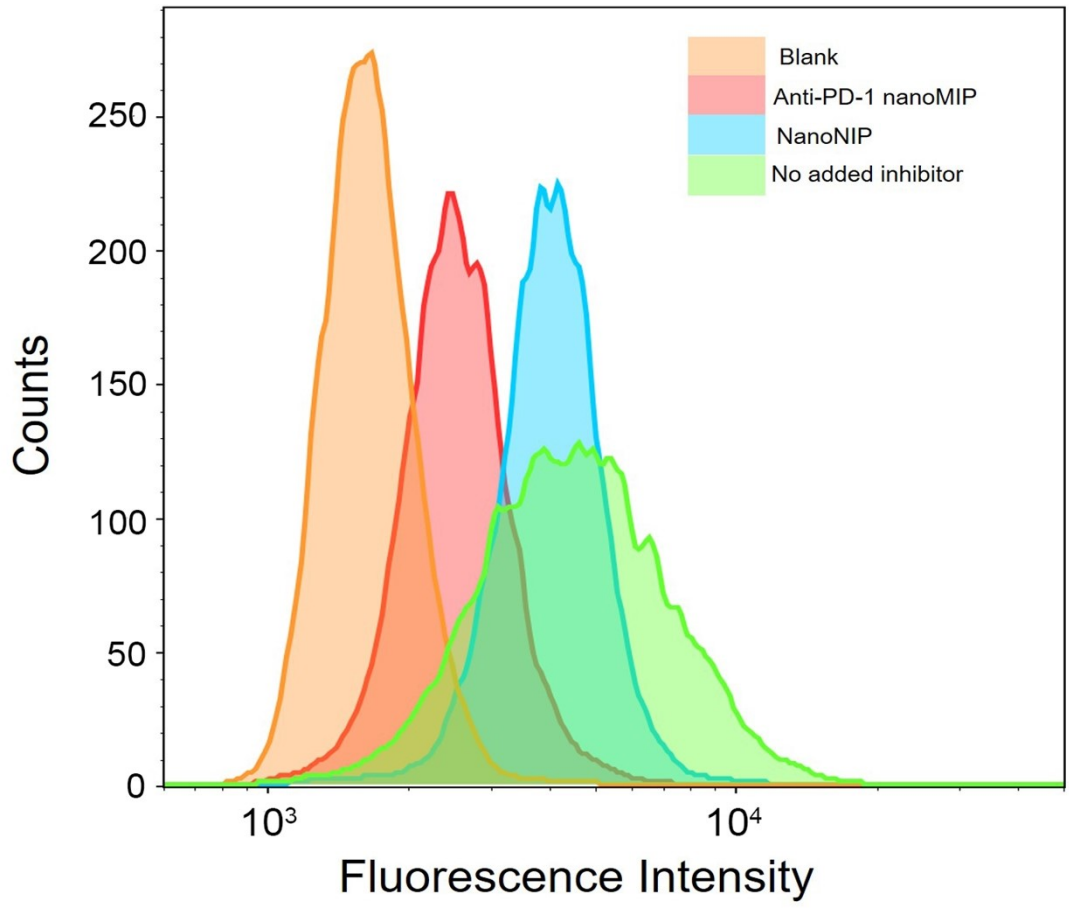



**Figure S9**. Anti-PD-1 nanoMIP blocked the PD-1/PD-L1 interaction on the cell surface. The concentration of anti-PD-1 nanoMIP and nanoNIP were 400 μg/mL.

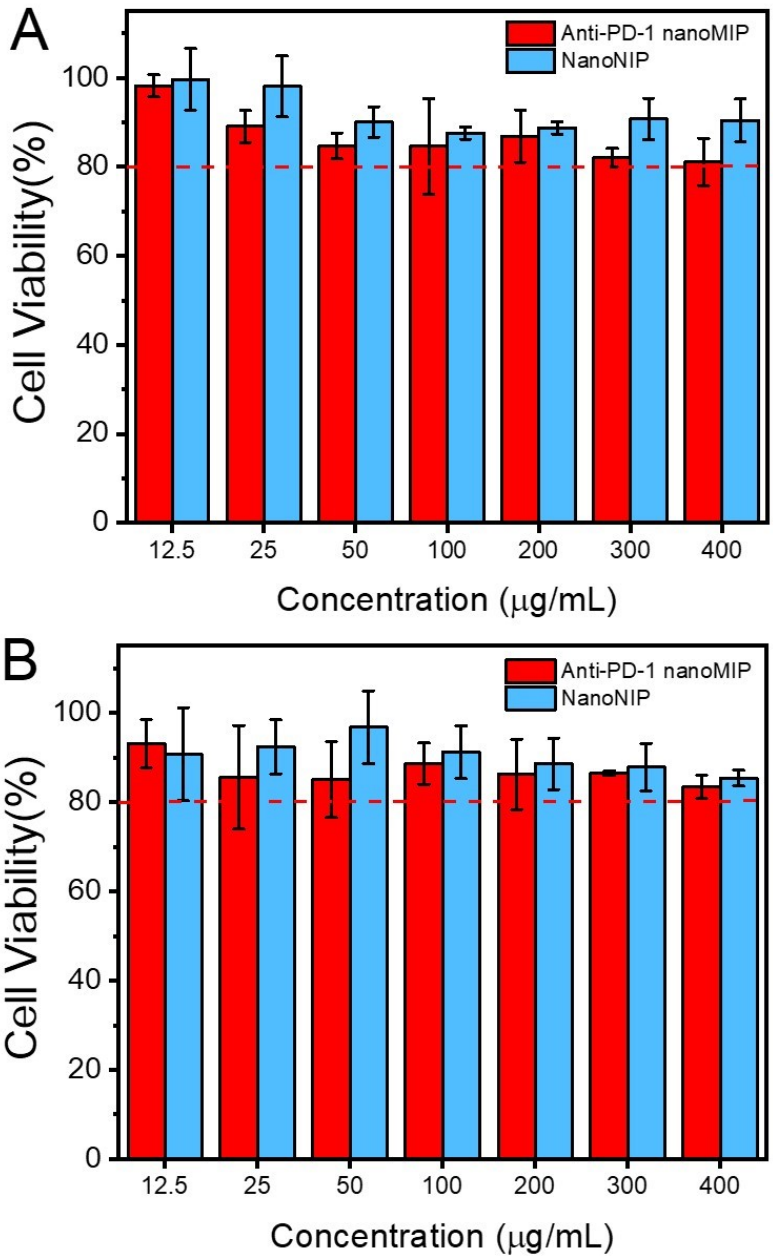



**Figure S10**. Cell viability of A) 786-O cells and B) Jurkat T cells after incubation with different concentrations of anti-PD-1 nanoMIP and nanoNIP for 24 hours. Data are presented as Mean ± SD (n = 3)

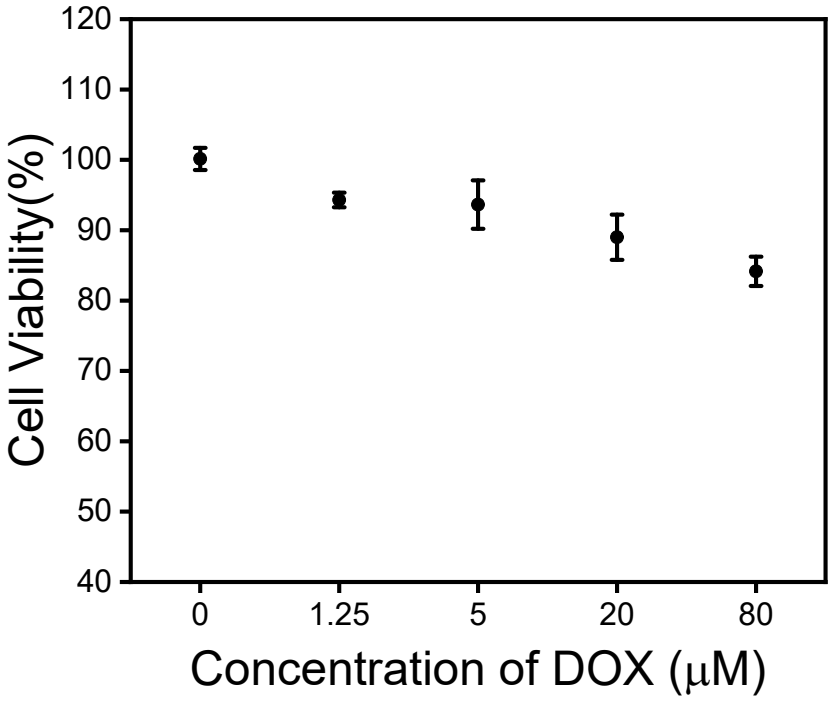



**Figure S11**. Dose-dependent chemosensitivity of 786-O cells exposed to gradient concentrations of DOX in a 786-O cells and Jurkat T cells co-cultured model. Data are presented as Mean ± SD (n = 3).

**Table S1.** Summary of the contents of the Si, C, O, N elements in anti-PD-1 nanoMIP.

| Atom | Surface Concentration (%) |
|---|---|
| Si2p | 22.10 |
| C1s | 14.42 |
| O1s | 60.67 |
| N1s | 2.81 |