Supporting Information

Dendritic Cells Reprogrammed by CEA messenger RNA loaded Multi-Functional Silica Nanospheres for Imaging-Guided Cancer Immunotherapy

Yue Hu [a,1], Yuying Tang [a,1], Xiao-Jing Zhang [a], Xiao-Tong Yang [a], Ying-Ying Tang [a], Su Li [a], Linyu Hu [a], Peng Chen [b] and Dong Zhu [a]**

[a] School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210023, P. R. China

[b] School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637457, Singapore

KEYWORDS: Immunotherapy, Reprogrammed dendritic cell, Silica nanospheres; Imaging-guided, Quantum dots

EXOERIMENTAL SECTION

Chemicals and Materials. Purified human CEA and its enzyme linked immunosorbent assay (ELISA) kit, astragalus polysaccharides (APS), lipopolysaccharide (LPS), DNase/RNase-free water, alamar blue (AB), DNA maker (DL 5,000) and fluorescein (FITC)-conjugated affinipure goat anti-rabbit IgG (H+L) were purchased from Nanjing Jin Yibai Biotech Co. Ltd (Nanjing, China). Linearized plasmid pcDNA3.1-CEA was customized by Wuhan Gene Create Biological Engineering Co. Ltd (Wuhan, China). Transcription kit (mMESSAGE mMACHINE® T7 Ultra Kit) was purchased from Thermo Fisher Scientific Company (Shanghai, China). Anti-CEA was purchased from Abcam plc (Shanghai, China). N-cetyltrimethyl ammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), cadmium chloride (CdCl2), mesitylene (TMB), mercaptosuccinic acid (MSA, 98%), sodium borohydride (NaBH4, 99%), sodium tellurite (Na2TeO3, 99.9%), 3-mercaptopropyl-trimethoxysilane (MPS, 99%), tri-sodium citrate dehydrate (99.3%), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 3-aminopropyltriethoxysilane (APS) were obtained from Aladdin. Cyclohexane (AR), n-pentanol (AR), ammonia solution (25wt %) and iso-propanol (AR) were purchased from the Shanghai Reagent Company. Poly-ethylene glycol tertoctylphenyl ether (Triton X-100) was acquired from Sangon Biotech Co. Ltd (Shanghai, China). Other chemicals were purchased from J&K Chemicals (Beijing, China). All these reagents were analytical grade and used as-received without further treatment. Ultrapure water (18.2 MΩ cm) prepared for all experiments was obtained from milli-Q-RO4 water purification system. (Millipore Simplicity MA, USA).

Cells. Dendritic cells (DCs) were generated from the bone marrow (BM) of 6- to 12-week-old C57BL/6 mice from BeNa Culture Collection Biotech Co. Ltd (Suzhou, China). The dendritic cell lines were introduced to mature according to Lutz method. Briefly, 2×10^6 DCs were seeded in each 100 mm bacteriological Petri dish with 10 mL of complete Iscove's modified medium (IMDM) supplemented with 10% fetal bovine serum (FBS), recombinant mouse GM-CSF (20 $ng\cdot mL^{-1}$, PeproTech) and mouse IL-4 (20 $ng\cdot mL^{-1}$, PeproTech), 100 $units\cdot mL^{-1}$ penicillin-streptomycin, and 2 mM L-glutamine. On day 3, an additional of 10 mL of the same medium was added into these dishes. On day 6 and day 8, half of the culture supernatant was centrifuged and collected, and then precipitation was suspended with media containing GM-CSF followed by re-adding into the original dishes. On day 10, suspended or loosely adherent cells were collected to obtain the immature DCs (imDCs) after blowing the medium gently. The generated imDCs were stimulated by various forms of antigen or/and nanoparticle complexes (MFNs@CEAmRNA or MFNs@CEA) as prepared and then induced to mature for further use by treating with LPS (1 $\mu g\cdot mL^{-1}$) for 18 h. The B16-F10 melanoma cell line was obtained from ATCC (American Type Culture Collection), and was cultured in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin in an incubator at 37 °C under 5% CO_2 atmosphere.

Mice. Eight-week-old male C57BL/6 mice (SPF level) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd and animal experiments were under protocols approved by Nanjing University of Chinese Medicine Animal Laboratory Center (permission ID: 201804A023). The tumour-bearing mice were humanely sacrificed when the diameter of tumours reached 2 cm.

Apparatus and characterization of nanoparticles. Transmission electron microscopy (TEM) and high-resolution TEM images were obtained using a FEI Tecnai F20 transmission electron microscope. The nitrogen sorption and desorption analysis was operated at 77 K using a micromeritics ASAP2020 sorptometer. The surface areas of nanoparticles were calculated by brunauer-emmett-teller (BET) method. UV-Vis absorption and fluorescence spectra of different nanoparticles were operated at PerkinElmer's VICTORTM X3 multimode plate reader. Agarose gel electrophoresis was recorded on a gel image analyzing system to identify mRNA transcription products.

Preparation of carcinoembryonic antigen mRNA(CEAmRNA). Linearized plasmid pcDNA3.1-CEA and PCR products that contain a T7 RNA polymerase promoter site were used as templates for in vitro transcription to obtain large amounts of efficiently and correctly CEAmRNA with mMESSAGE mMACHINE® T7 Ultra Kit. Poly (A) tailing reagents in the mMESSAGE mMACHINE® T7 Ultra Kit were used to add a ⩾50-100 base poly (A) tail to the RNA transcripts. The resulting capped and tailed RNA can then be used in transfection or microinjection experiments, where enhanced translation over untailed mRNAs may be seen in virtue of increased mRNA stability and translation efficiency. The reaction products were purified by Lithium Chloride (LiCl) precipitation method according the kit process and then quantitated by UV light absorbance. Subsequently, identification of transcription products was carried out by agarose gel electrophoresis and dissolved in DNase/RNase-free water to form a stock solution (1.0 μg μL-1) for further experiments.

Preparation of microkernel-based (SiO_2-CdTe-SiO_2)@SiO_2 mesoporous fluorescent nanoparticles. The (SiO_2-CdTe-SiO_2)@SiO_2 nanoparticles were fabricated by designing a multiple-layer mesoporous nanostructure composed of a protective silica coating CdTe QDs core

and a mesoporous silica shell according to previous report with modification. Briefly, the thiol-functionalized silica core was firstly synthesized. Cyclohexane (7.0 mL), ammonia solution (25 wt %, 220 μL), n-pentanol (1.70 mL), Triton X-100 (1.65 mL) were added 400μL of ultrapure water in a flask and sealed under stirring for 30 min. Then 100μL of TEOS was added and reacted for 3 days. After that MPS (10 μL) was added for another 24 h. Isopropanol was used to terminate the reaction, and the prepared precipitate was centrifuged, washed with isopropanol, ethanol, and water, respectively, and dispersed in pure water to obtain the silica core. Subsequently, 50 mL of 0.04 mol • L^{-1} $CdCl_2$, 800 mg of trisodium citrate dehydrate, and a silica core were respectively added into a silica core solution in a three-necked flask and stirred for 15 min. Then 0.2 g of MSA was added into the above solution, and the pH was adjusted to 10.5. Under the nitrogen flow, NaBH4 (0.1 g) and 10 mL of 0.01 mol·L^{-1} Na_2TeO_3 were added, refluxing at 100 °C for 24 h. The sample was centrifuged and washed with ethanol to give core-satellite SiO_2@CdTe suspensions.

Finally 0.5 g of CTAB and 3.5 mL of TMB were added into SiO_2@CdTe suspensions, 2 mL of 2 mol • L^{-1} NaOH, stirring at 80 °C for 4 h. TEOS (2.5 mL) was quickly introduced into the solution for another 2 h reaction. The resultant precipitate was centrifuged, washed, and dried in a vacuum at 45 °C. The dried precipitate was refluxed in an ethanol solution (NH4NO3/C2H5OH, 10 mg·mL^{-1}) to remove the residues. Then, the products were aminated by APS under refluxing in anhydrous ethanol at 80 °C and the resultant products were filtered, washed, and dried to obtain amino group functionalized MFNs.

Immobilization of CEAmRNA and CEA onto the MFNs. CEAmRNA (20μL at 1 μg • $μL^{-1}$ concentration) in DNase/RNase-free water solution was mixed into the MFNs solution (weight ratio of CEAmRNA:MFNs was 1:30) to form CEAmRNA nanocomplexes, which were kept for

30 min at 200 rpm. stirring at room temperature to stabilize. The MFNs exhibit positive charge due to the presence of amino-groups on the surface. By electrostatic adsorption, the MFNs could absorb CEAmRNA solution. The MFNs@CEAmRNA were then washed three times with ice-cold Hypure water and finally concentrated into 500μL solution.

CEA was immobilized on the surface of amino-functionalized MFNs by the typical amidation reaction activated by EDC/NHS. In detail, a 10 mg portion of MFNs was rinsed with 2 mL of potassium phosphate buffer (10 mM, pH 5.5) three times. Then, CEA (20 μg) was suspended in 40 μL of potassium phosphate buffer (10 mM, pH 5.5) and the suspension was added to the MFNs followed with vortex-mixing for 5 min. Finally, 20 μL of EDC-NHS solution was introduced into the mixture and oscillated with rotation at 200 rpm for 24 h at 4 °C. When the system turned back to room temperature, the mixture was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. The resultant products, i.e.MFNs@CEA, were washed three times with 5 mL of Tris-HCl buffer (10 mM, pH 7.4) and then dried with nitrogen for further use. The MFNs@CEAmRNA and MFNs@CEA were used fresh or kept at -80 °C to use later for various in vitro and in vivo studies

Cell viability assay. Cell viability was measured by the cell counting kit-8 (CCK-8) assay (Solarbio). The generated imDCs (1×10^5 cells/well) were seeded in a 96-well micro plate and then incubated with 10 μL of various concentrations of nanocomposites, viz., 5, 10, 25, and 50 $\mu g \cdot mL^{-1}$ for 24 h. After incubation, 10 μL of CCK-8 solution was added to each well for another 2.5 h at 37 °C. The absorbance of each sample at 450 nm was measured using multimode plate reader (Tecan).

Lymphocyte proliferation assay. The MFNs@CEA-treated DCs, MFNs@CEAmRNA-treated DCs and free DCs (1×10^6 cells) through subcutaneous injection into the hind-leg footpads, which

were divided into nine groups. After immunizing the C57BL/6 mice with different DCs twice at weekly intervals, lymphocytes harvested from periaortic, leg fossa and superficial inguinal lymph node were cultured for 3d in complete medium supplemented with 10% FBS and indicated concentrations of purified CEA proteins in flat-bottomed 96-well culture plates (1×10^4 cells/well). The cells were labelled with Alamar Blue in different time intervals for optimum incubation time. Lymphocyte proliferation was measured using the Alamar Blue assay according to the manufacturer's protocol and a microplate reader (TECAN). Absorbance was read at the wavelength of 570 nm and 600 nm. Cell proliferation was recorded as the ratio between mean of lymphocyte in immunized animals and mean of lymphocyte in control animals (treated with PBS) (stimulation index, SI). SI= $A_{570\ nm}$ with samples/ A570 nm with control, where A_{570} = Absorbance at 570 nm. All samples were assayed in triplicate. Spleens from immunized mice were obtained for further analysis.

Cytotoxic T lymphocyte (CTL) activity. The cytotoxic responses of effector cells were assessed by performing the Alamar Blue test as described previously. Briefly, splenocytes harvested from mice were restimulated with 20μg • mL^{-1} purified CEA for 3 d in complete medium to obtain effector cells. The cells were washed with PBS to remove residual proteins completely and then cultured with B16-F10/CEA or B16-F10 target cells in flat-bottomed 96-well culture plates at 20:1 ratio (effector: target). After 18 h of incubation, mixed cells and single effector cells or target cells were incubated with Alamar Blue at 37 °C for the same time. After appropriate incubation time, fluorescence value was measured by a microplate reader with emission at 590 nm. The percentage of specific lysis was calculated according to: % specific lysis = (fluorescence value (FL) targets + FL effectors – FL(effectors +targets))/FL targets × 100.

Tumor immunotherapy. Six-week-old C57BL/6 mice were subcutaneously injected at the right hind flank with B16-F10-CEA cells (1.5×10^5 cells/mouse). At 7 d after tumor cell injection, the mice were divided into nine groups (n=5) and immunized at the tail base with DCs (1×10^6 cells/mouse) stimulated with indicated nanoparticles or antigens three times every six days. Tumors were measured by digital caliper (AIRAJ), and the tumor volume (mm^3) was calculated as $(A \times B^2)/2$, in which A is the long diameter and B is the short diameter.

Confocal imaging. To study the localization of MFNs@CEA or MFNs@CEAmRNA in imDCs, cell imaging was conducted by confocal laser scanning microscope (CLSM). MFNs@CEA or MFNs@CEAmRNA was dispersed in complete medium and filtered to form a 25 $\mu g \cdot mL^{-1}$ stocking solution of nanoparticles. The generated imDCs were seeded at 5000 cells/well in a sterilized single-well confocal dish overnight and then cultured by stocking solutions of the nanoparticles for 2 h in an incubator at 37 °C under 5% CO_2 atmosphere, fixed with paraformaldehyde for 15 min, and then stained with DAPI for 5 min. Confocal fluorescence imaging of DCs was performed on Nikon TI-E-A1R confocal laser scanning microscope with excitation at 488 nm.

Immunofluorescence staining and microscopy. Immunofluorescence staining for CEA *in vitro* expression study was performed on Axio Vert A1 inverted microscope. For immunofluorescence staining, imDCs treated by different nanocomposites loaded with antigen or CEAmRNA were plated onto coverslips in 24-well plates, washed with ice-cold complete culture for three times and fixed with 4% paraformaldehyde for 30 min at 37 °C. Cells were then washed with ice-cold PBS and permeabilized by incubation in 0.1% Triton X-100-PBS for 10 min, followed by blocking with PBS blocking buffer containing 1% BSA for 1 h at room temperature. Then, the samples were incubated in primary antibody (1:200 anti-CEA antibody) overnight at 4 °C,

washed with PBS and incubated in goat anti-rat-FITC at 1:50 dilution in blocking buffer for 2 h at 37 °C. Finally, stained cells were washed with PBS in the dark, counterstained with DAPI, and mounted on slides.

Flow cytometry assay. Immature DCs were counted by using a hemo-cytometer and then seeded in a 6-well micro plate at 1×10^6 cells/mL in complete medium, followed by incubation with different nanocomposites at 37 °C in 5% CO_2 for 18 h. To measure the maturation level of DCs after various treatments, as-treated DCs were washed with FACS buffer (PBS solution containing 1% FBS) and subsequently stained with anti-CD80-APC, anti-CD11c-FITC and anti-CD40-PE antibodies (eBioscience) for 30 min at room temperature in the dark. Finally, these DCs were washed again with FACS buffer and then analyzed by flow cytometry for intracellular markers detection.

Tumor growth. Six-week-old C57BL/6 mice were subcutaneously injected at the right hind flank with B16-F10-CEA cells (1.5×10^5 cells/mouse). At 7 d after tumour cell injection, the mice were divided into nine groups (n=5) and immunised at the tail base with DCs (1×10^6 cells/mouse) stimulated with indicated nanoparticles or antigens three times every six days. Tumours were measured by digital caliper (AIRAJ), and the tumour volume (mm^3) was calculated as $(A \times B^2)/2$, in which A is the long diameter and B is the short diameter.

In vivo imaging. For *in vivo* DC tracking, a C57BL/6 mouse was injected *s.c.* at the hind-leg footpad with MFNs@CEAmRNA- reprogrammed DCs (2×10^6 cells), while the other mouse was injected *s.c.* with MFNs@CEAmRNA nanoparticles (20 μL, 50 $\mu g \cdot mL^{-1}$) as control. After 48 h, mice were then anesthetized for whole-animal fluorescence imaging.

Tissue fluorescence imaging. All the tumour-bearing immunized mice from the treatment groups were sacrificed, six days after the last caudal injection. The tumour masses and major organs (liver, spleen, kidney, and lung) from these mice were collected, fixed in 4% neutral buffered formalin, embedded in paraffin and cut into histological tissue sections for fluorescence imaging and biodistribution analysis by optical microscope.

Statistical analysis. All graphs were prepared using GraphPad Prism 7 software, and statistical analyses were also carried out using GraphPad Prism 7 software to perform one-way analysis of variance (ANOVA), two-sided t-tests. All experiments were performed in triplicate unless otherwise stated. Error bar indicate the standard deviation (SD) unless otherwise noted specifically as the standard error of the mean (SEM). All significant values shown in various figures are presented as mean ± standard error and considered statistically significant at $P < 0.05$.

1. The characteristics of MFNs.

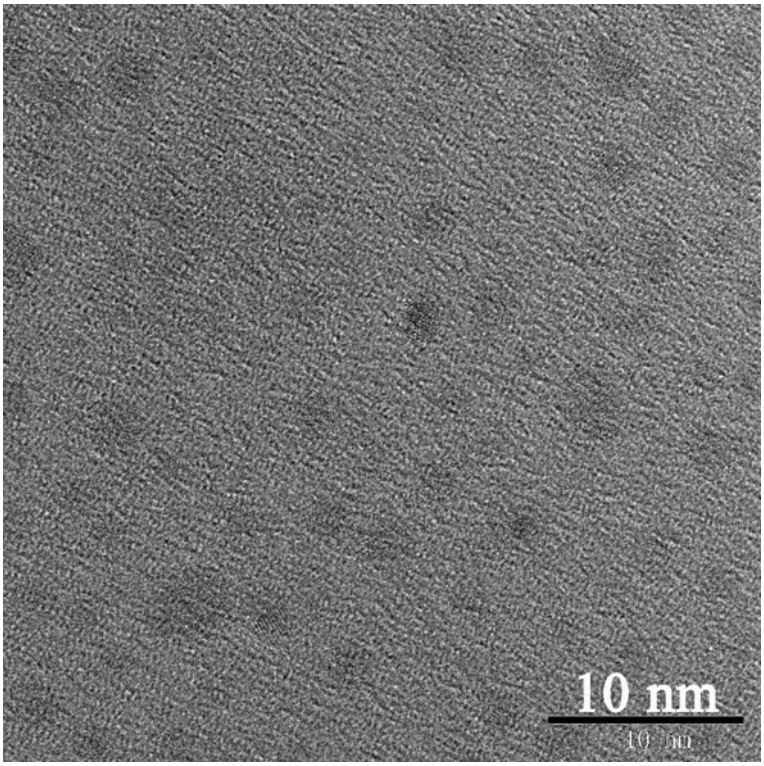


Figure S1. TEM image of thiol-functionalized silica nanoparticles as the innermost core.

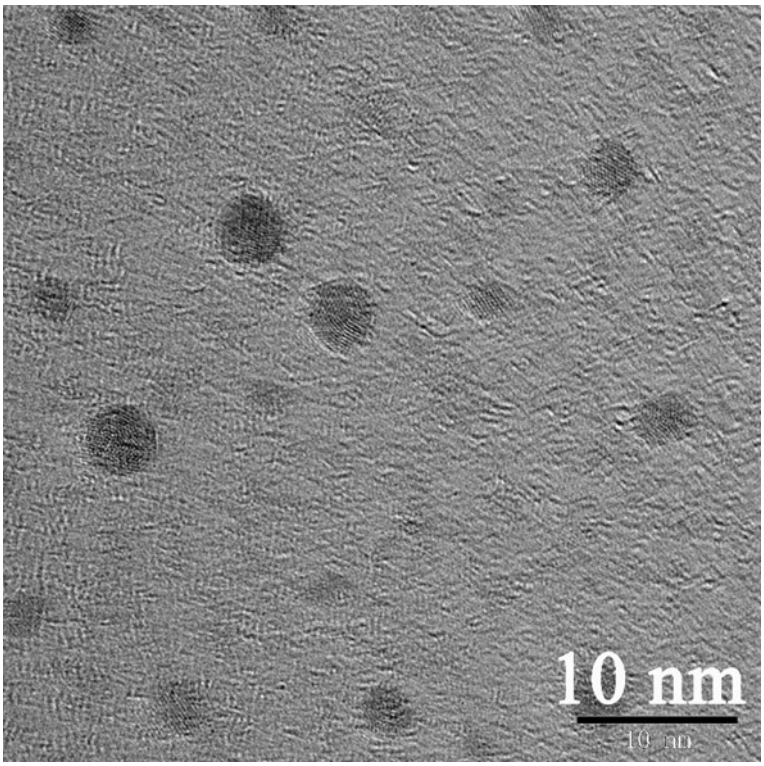


Figure S2. TEM image of SiO_2@CdTe nanoparticles.

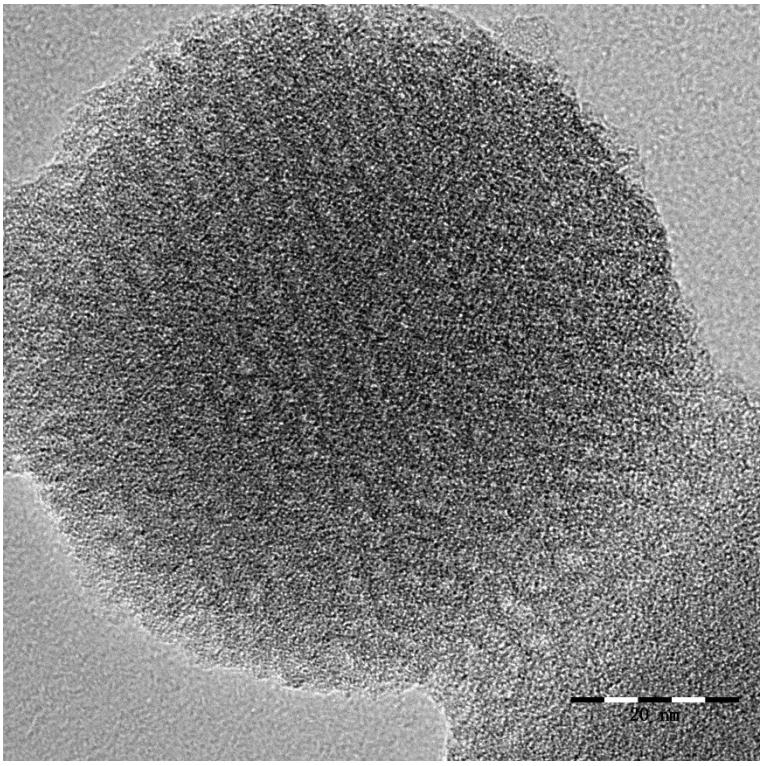


Figure S3. High-resolution TEM image of MFNs.

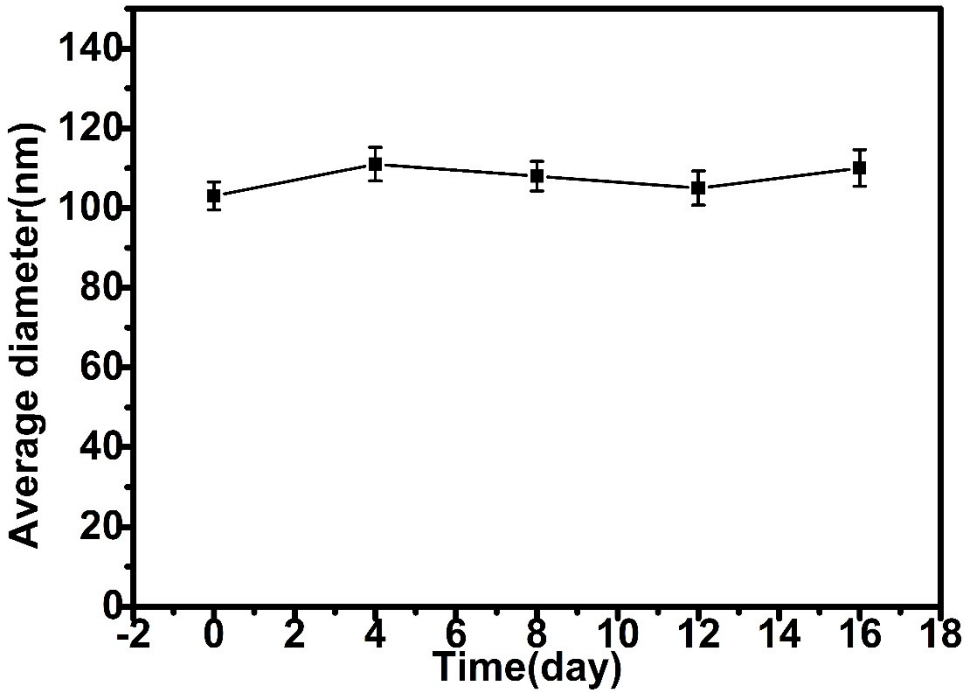


Figure S4. Hydrodynamic diameter of MFNs@CEAmRNAs after different days of storage in serum.

2. **Preparation of CEAmRNA.**

Plasmid pcDNA3.1(+) was designed as a vector for *in vitro* transcription of CEA cDNA, and both terminus of the CEA cDNA were digested and inserted into the downstream of T7 promoter for combining T7 RNA polymerase to initiate the transcription (Figure S3A, Supporting Information). The sequence mapping confirmed that the inserted DNA fragment was identical to the sequence of mRNA coding region of human CEA gene (Figure S4, Supporting Information). The constructed recombinant plasmid pcDNA3.1-CEA can be digested into two bands by restriction endonucleases (Kpnl and EcoRI), and the smaller fragment has the same length as with the inserted fragment containing 2109 base pair (bp) as shown in Figure S3B, indicating that CEA cDNA had been correctly inserted into pcDNA3.1(+) vector. MESSAGE mMACHINE® T7 Ultra Kit was then utilized for in vitro transcription and the transcription products were identified by agarose gel electrophoresis.

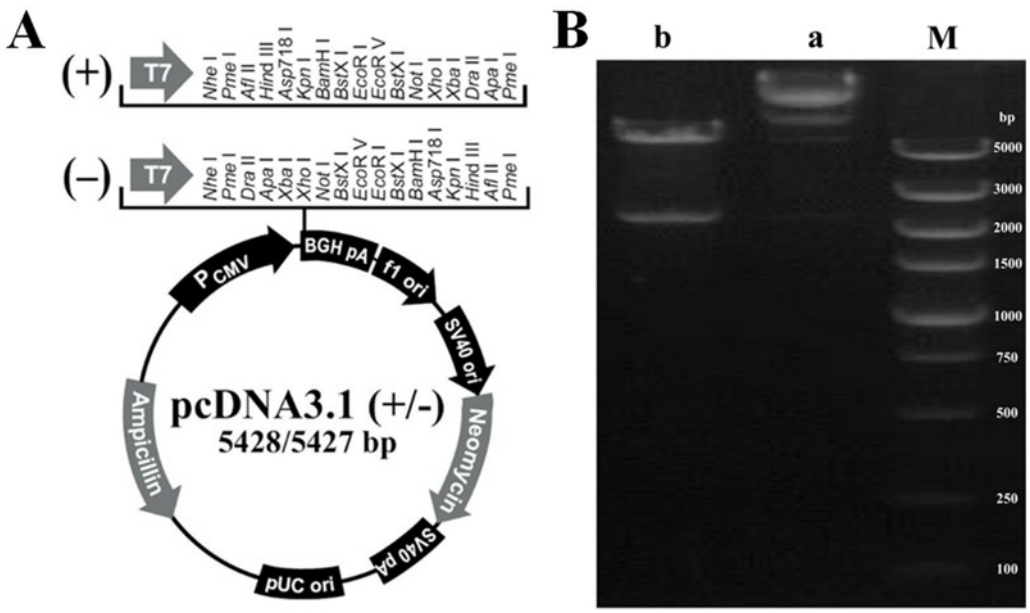
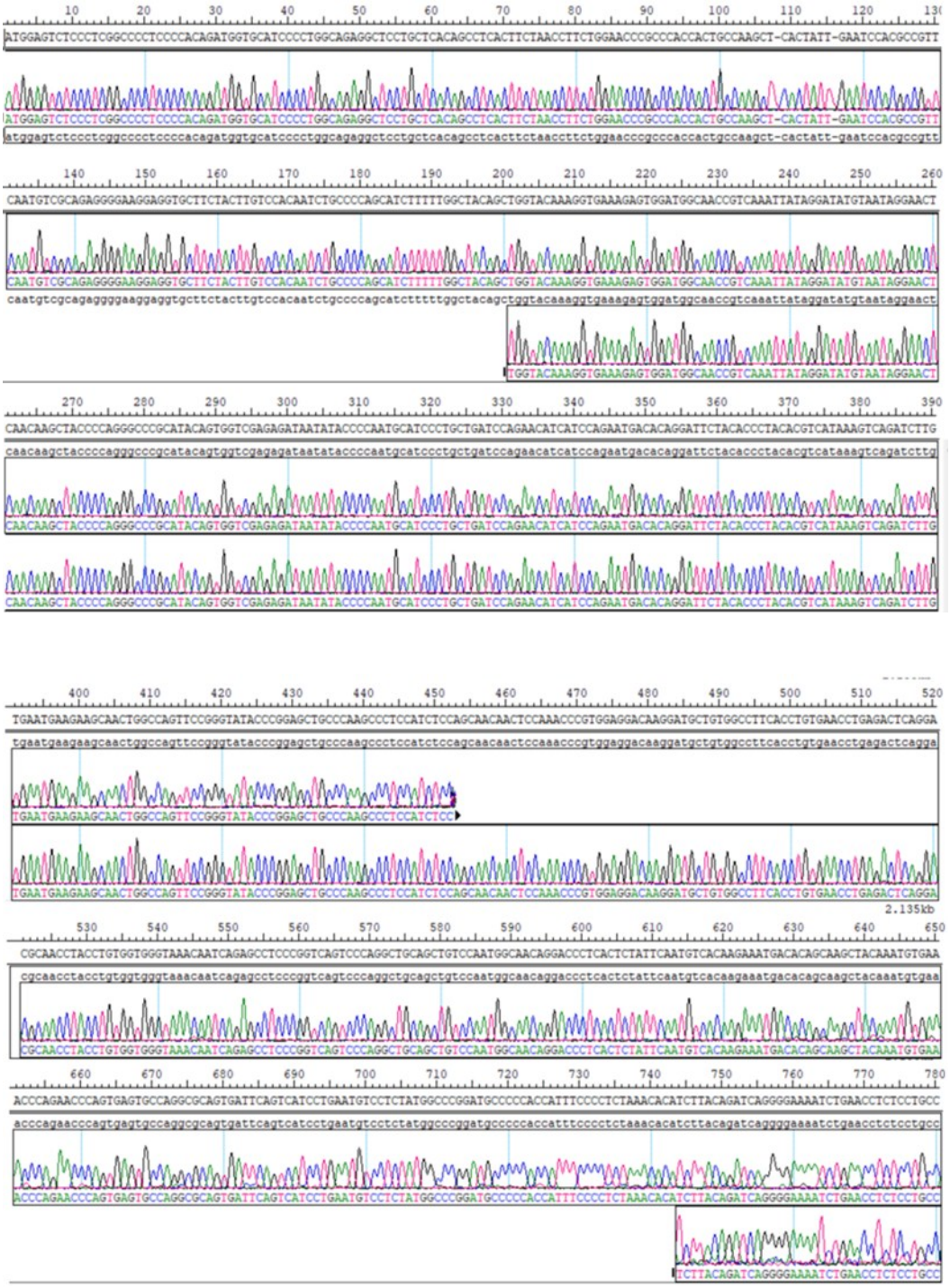


Figure S5. A) Illustration of plasmid pcDNA3.1-CEA and multi-cloning sites. B) Restriction digestion map of pcDNA3.1-CEA. M: DNA Marker. a: Plasmid digested by Kpnl and EcoRI. b: Plasmid DNA.



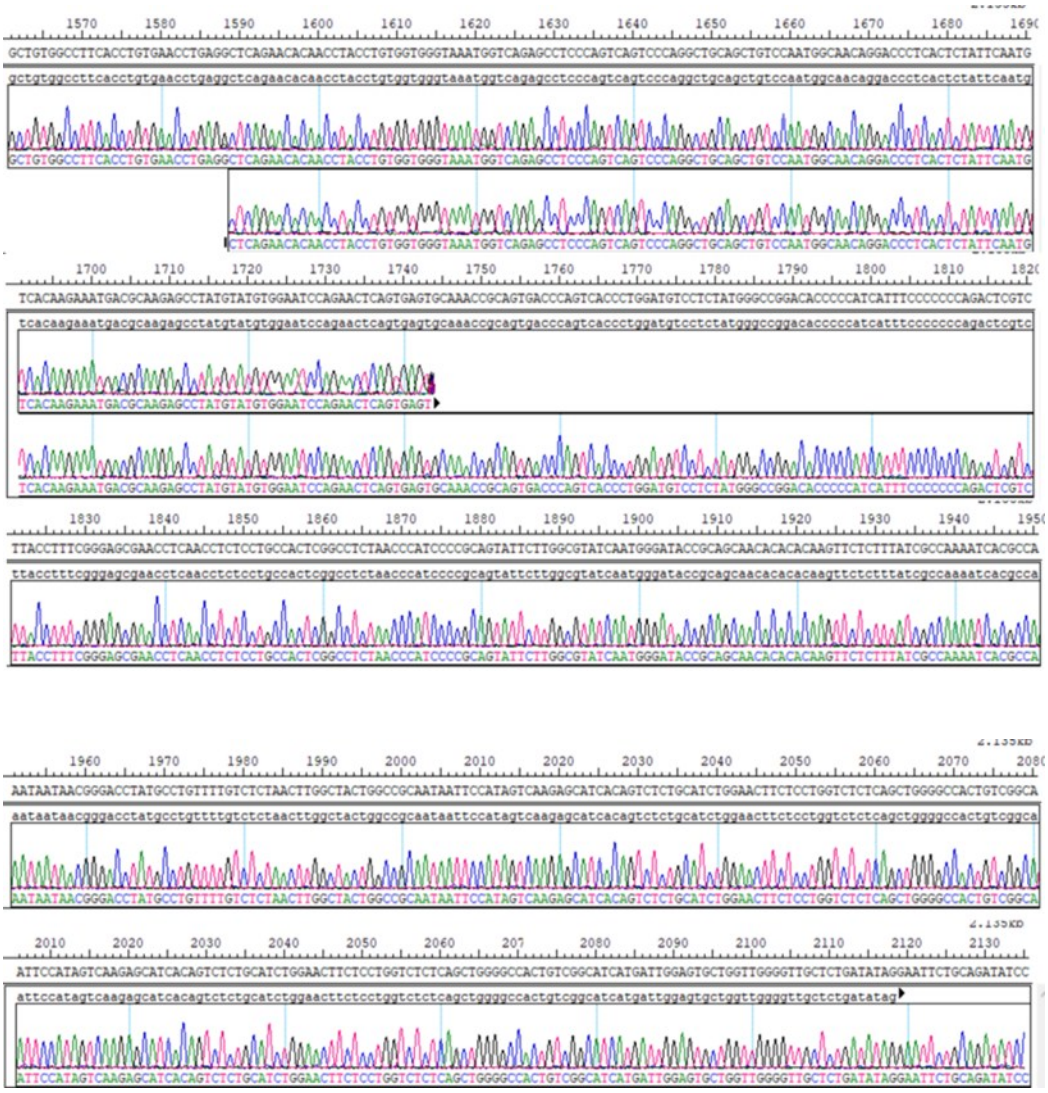


Figure S6**.** Sequence map of recombinant plasmid pcDNA3.1-CEA.

3. Morphological features of DCs

The morphological features of DCs maturation at different stages were first assessed through an optical inverted microscope as shown in Figure S5. Incipient bone marrow DCs were round or small elliptic adherent cells. After treatment by inducers such as GM-CSF and IL-4, they slowly became colony-grown suspension cells, indicating the tendency of going maturate.

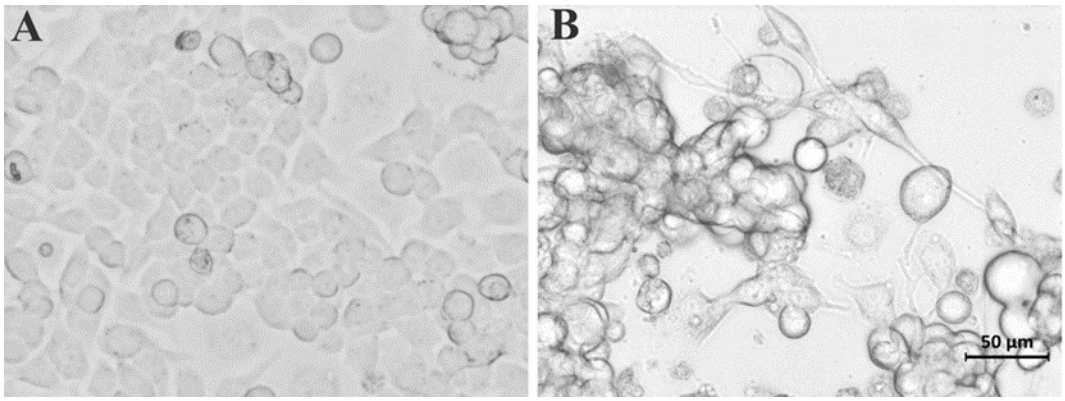


Figure S7**.** Morphological features of DCs at different stages in optical inverted microscope (Scale bar: 50 μm, 400×). A) BMDCs are round or small elliptic adherent cells. B) Immature DCs are colony-grown roundish cells with rare if any long extensions treated by GM-CSF and IL-4.

4. Cell viability of imDCs incubated with different nanoparticles.

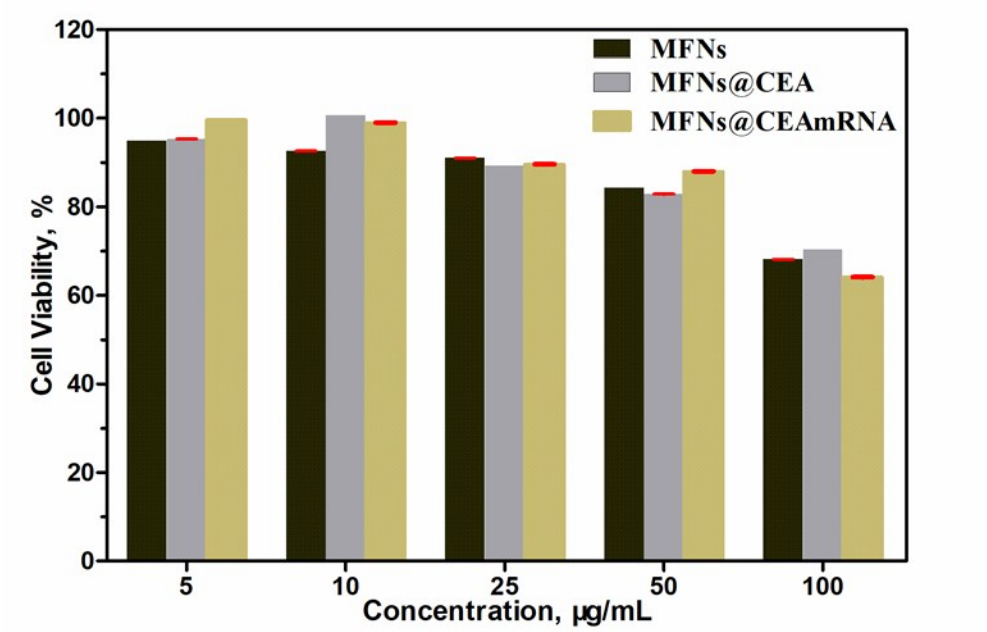


Figure S8. Cell viability of imDCs after incubation of different nanoparticles at different concentrations.

5. Optimization for T lymphocyte proliferation assay.

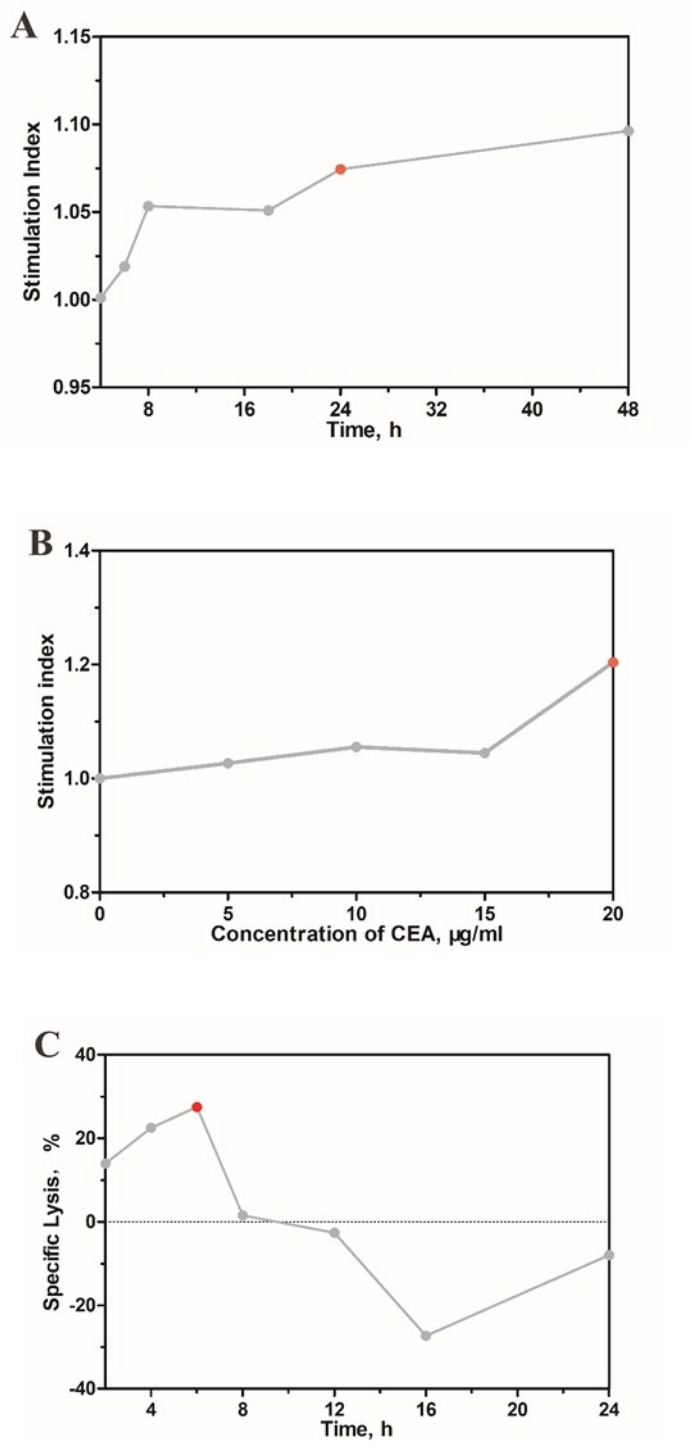


Figure S9. Optimization of Alamar Blue test. A) Optimal incubation time for T cell proliferation capability. B) Optimal pre-stimulated CEA concentration for immune responses. C) Optimal incubation time for cytotoxic T lymphocyte activity.