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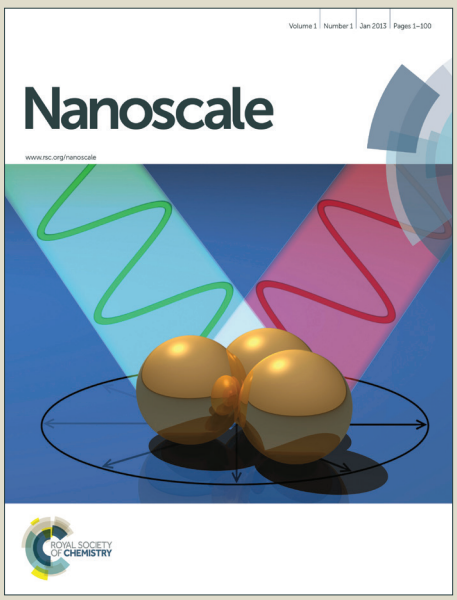





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# Gold nanoclusters-based vaccines for dual-delivery of antigens and immunostimulatory oligonucleotides

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We here report a facile one-pot synthesis of fluorescent gold nanoclusters (AuNCs) via the peptide biomineralizatio method, which can elicit specific immunological responses. The as-prepared peptide-protected AuNCs (peptide-AuNC display strong red fluorescence, and more importantly, as compared to peptide alone, the immune stimulatory ability of the resulted peptide-AuNCs can not only be retained, but also can be efficaciously enhanced. Moreover, through dua delivery of antigen peptides and cytosine-phosphate-guanine (CpG) oligodeoxynucleotides (ODNs), the as-prepared peptide-AuNCs-CpG conjugates can also act as smart self-vaccines to assist in generation of high immunostimulatory activity, and be applied as the probe for intracellular imaging. Both in vitro and in vivo studies provide the strong evide that the AuNCs-based vaccines may be utilized as safe and efficient immunostimulatory agents that are able to prevent and/or treat a variety of ailments.

## 1. Introduction

Vaccines against infectious diseases are one of the greatest medical inventions in human history,[1-6]as they are highly effective therapeutic strategies for preventing various diseases,[7-9] which may lead to greatly improved healthcare and dramatically decreased mortality.[10]The search for safe and effective vaccines has been ongoing for more than 1000 years since human beings began fighting diseases through vaccination.[11]Traditional vaccines utilizing live attenuated pathogens or inactivated (killed) viruses have been widely employed for the treatment and prevention of a variety of diseases since the beginning of the vaccinology era, and vaccines against infectious diseases including influenza, polio, malaria, smallpox, yellow fever, and hepatitis B, etc. have been applied to immunize billions of people worldwide.[12] Although conventional live vaccines-based therapeutic strategies have generated successful results for the treatment of a variety of diseases, safety concerns and manufacturing difficulties may impede their further wide biomedical applications.[13, 14]Recent endeavors to develop safe and effective vaccines have focused on the constructing of subunit vaccines, which are commonly composed of highly purified recombinant antigens, like proteins and peptides.[15-17]These synthetic subunit vaccines provide distinct benefits, such as decreased cost, enhanced safety, ease of manufacture, and capability of stimulating the antigen specific immune response, over the conventional vaccines.[18-20]Despite their potential advantages, general shortcomings of subunit vaccines, including the poor and short-lived immunogenicity, inefficient uptake by professional antigen presenting cells (APCs), as well as fast degradation by metabolic enzymes still seriously limit their further applications in vaccine research.[21-24]Alternatively, nanotechnology provides scientists with a robust platform for the improvement of the immunity of subunit vaccines.[22, 23, 25-

On the other hand, gold nanomaterials have been extensively investigated for biomedical applications due to their good biocompatibility, high chemical stability, and facile synthetic procedure.[28-31]For example, as a novel platform for vaccine development, gold nanomaterials have been broadly applied for vaccine antigens or adjuvants delivery. In 2012, Fan and Huang claimed immune enhancement via adjuvant CpG oligodeoxynucleotides (ODNs) delivered to macrophage cells by gold nanoparticles (AuNPs).[32, 33] After that, Jon and co-workers also used the AuNPs as a platform for vaccine development: the AuNPs serve as a scaffold for antigen and adjuvants delivery, which enabled effective cancer prevention and therapy.[34, 35]In addition, gold nanoclusters (AuNCs), which consist of a few to roughly a hundred atoms and have sizes comparable to the Fermi wavelength of an electron, have also attracted increasing attention in recent years.[36] As compared to gold nanoparticles which show strong surface plasmon absorption, AuNCs with sizes below2 nm exhibit molecule-like properties including size dependent fluorescence[37-39] and discrete electronic states.[40-43] The attractive fluorescence properties of AuNCs prompt a wide interest in developing AuNCs-based sensors for biological detection and probes for bioimaging.[44-49] Recently, our group has also developed a fac

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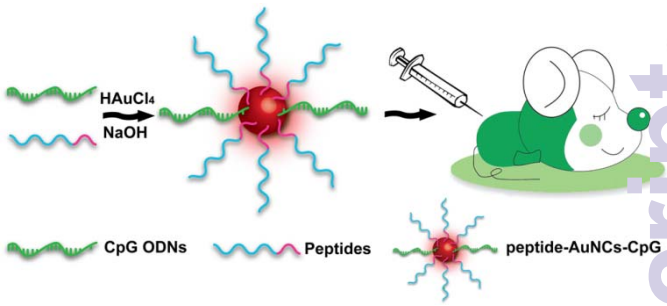
one-pot synthesis of self-vaccine AuNCs by using ovalbumin (OVA)-CpG conjugates as the templates, which can elicit specific immunological responses through dual-delivery of protein antigen and CpG ODNs into the same antigen presenting cells (APCs).[50]Although great progress has been achieved in this field, the general limitation of current synthetic subunit vaccines is that they suffer from the inability to elicit strong $CD8^+$ cytotoxic T lymphocyte(CTL) responses, which are crucial in chronic viral diseases, such as HIV and hepatitis C, as well as cancer immunotherapy.[26, 51-53]Therefore, there is an urgent need to develop effective vaccines that can mount strong $CD8^+$ T cell mediated immunity in order to apply them in clinical applications. OVA peptide SIINFEKL is an octameric peptide from ovalbumin presented by the class I major histocompatibility complex (MHC) molecule.[54-56]It has been demonstrated that peptide SIINFEKL can be used to induce strong $CD8^+$cytolytic T cell responses.[52, 57-59]

Here we report the facile one-pot synthesis of fluorescent AuNCs via the peptide biomineralization method, combining attractive features of enhanced immunological responses and intracellular imaging (Scheme 1). This approach relies on the design of the functionalized peptides, $H_2N$–CCYSIINFEKL–COOH (abbreviated as CCYSIINFEKL), that contain two different domains: domain one is composed of CCY that can generate highly fluorescent AuNCs, represented by the red line, domain two is OVA peptide SIINFEKL, which is able to effectively stimulate the immune responses, represented by the blue line. The as-prepared peptide-AuNCs display strong red fluorescence, and more importantly, as compared to peptide alone, the immunostimulatory ability of the resulted peptide-AuNCs can not only be retained, but also can be efficaciously enhanced. Moreover, the thiolated CpG ODNs can further be involved in the synthesis of AuNCs (peptide-AuNCs-CpG conjugates). The resulting peptide-AuNCs-CpG conjugates are able to effectively deliver the antigen and the adjuvant CpG ODNs to the same antigen-presenting cells (APCs), which are critical to induce strong and long-term immune responses.[60, 61]In addition, the capacities of increasing the CpG and peptide stability against nuclease degradation and enhancing CpG and peptide uptake ability by TLR9-positive cells also endow the peptide-AuNCs-CpG conjugates with the ability to act as self-vaccines to assist in generation of high immune response. Both in vitro and in vivo studies provide the strong evidence that the peptide-AuNCs-CpG conjugates can induce strong immune responses, highlighting the potential of the peptide-AuNCs-CpG conjugates to serve as promising self-vaccines for immunotherapy. Significantly, the fascinating optical property also indicates that the peptide-AuNCs-CpG conjugates are promising optical probes for fluorescent imaging. Compared with our previous work,[50] highlights of this work are to (i) avoid the covalent modification process and simplify the synthesis of AuNCs, as well as (ii) elicit strong $CD8^+$CTL responses. To the best of our knowledge, our method is the first example using the peptide functionalized AuNCs that are able to serve as both fluorescent probes for cell imaging and a potent platform for immunotherapy.




**Scheme 1.**General scheme for the synthesis of the peptide-AuNCs-CpG conjugates to induce immune response.

## 2. Experimental section

### 2.1. Materials and Instrumentation

Purified anti-mouse TNF-α, biotinconjugated anti-mouse TNF-α cocktail, TNF-α standard, anti-mouseIL-6, biotin anti-mouse IL-6, IL-6 standard, anti-mouse IFN-γ, biotinconjugated anti-mouse IFN-γ and IFN-γ standard were purchased from eBioscience. Peptide CCYSIINFEKL (Purity: 97.6%) was purchased from GL Biochem Co., Ltd. (Shanghai, China).OPD (o-phenylenediamine) substrate was obtained from DingGuo. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen. The mouse leukemic monocyte macrophage cell line(RAW264.7 cell line) was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai). $HAuCl_4$ and all other reagents were of analytical reagent grade, and used as received. Nanopure water (18.2 MΩ; Millpore Co., USA) was used in all experiments and to prepare all buffers. Sulfydryl tagged CpG ODNs (5′-TCC ATG ACG TTC CTG ACGTT-SH-3′) were synthesized by Sangon Biotechnology Inc. (Shanghai, China).

Atomic-force microscopy (AFM) measurements were performed using Nanoscope V multimode atomic force microscope (VeecoInstruments, USA). Tapping mode was used to acquire the images under ambient conditions. Transmission electron microscopy (TEM) images were recorded using a FEI TECNAI G2 20 high-resolution transmission electron microscope operating at 200 kV. Fluorescence measurements were carried out on Jasco-FP-6500 spectra fluorometer (Jasco International Co. LTD. Tokyo, Japan). UV/Visible absorption spectra were recorded using a Jasco-V550 UV/Vis spectrophotometer. Circular dichroism spectra were recorded on a JASCOJ-810 spectropolarimeter. EDS was carried out using a HITACHI S-4500 instrument. Fluorescence images were captured using an Olympus BX-51 optical equipped with a CCD camera.

### 2.2. Synthesis of the peptide-AuNCs

All glassware was washed with aqua regia, and then rinsed with ultrapure water and ethanol. In a typical experiment, an

aqueous solution of $HAuCl_4$ (22.5 mM, 19.5 μL) was slowly added to a solution of peptide CCYSIINFEKL (1.06 mM, 410μL) in a 5 mL vial under vigorous stirring, and then NaOH (1 M, 9μL) was added within 30 seconds to give a final pH of ~12. The sample was sealed and stored in the dark for 12 hours without any disturbance to produce the peptide-AuNCs. Then the as-synthesized peptide-AuNCs were dialyzed by a dialysis tube (MWCO: 3000) to remove free peptide and unreacted reagents. And the concentration of the peptide was adopted to confirm the concentration of the peptide-AuNCs in the following experiments.

### 2.3. Synthesis of the peptide-AuNCs-CpG Conjugates

The sulfydryl-tagged CpG ODNs were reduced with excess Tris-(2-carboxyethyl) phosphine (TCEP)at room temperature for 1 h, and residual reagents were removed by the dialysis tube (MWCO: 3000).In a typical experiment, an aqueous solution of $HAuCl_4$ (22.5 mM, 19.5 μL)was slowly added to the mixture solution of peptide CCYSIINFEKL (2.07mM, 210μL) and the resulting sulfydryl-tagged CpG ODNs (217 μM, 200μL) in a 5 mL vial under vigorous stirring, and then NaOH (1 M, 9μL) was added within 30 seconds to give a final pH of ~12. The sample was sealed and stored in the dark for 12 hours without any disturbance to produce the peptide-AuNCs-CpG conjugates. Then the as-synthesized peptide-AuNCs-CpG conjugates were dialyzed by a dialysis tube (MWCO: 10000) to remove free peptide, CpG ODNs and unreacted reagents. And the concentration of the peptide was adopted to confirm the concentration of the peptide-AuNCs-CpG conjugates in the following experiments.

### 2.4. Cell Culture

The murine macrophage-like RAW264.7 cells were grown at 37°C in an atmosphere of 5 v/v% $CO_2$ in air, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS,1.5 g $L^{-1}$ $NaHCO_3$, 100 units $mL^{-1}$ penicillin, 100 mg $mL^{-1}$ streptomycin,4.5 g $L^{-1}$ glucose and 4 mM glutamine. The media was changed every three days, and the cells were digested by trypsin and resuspended in fresh complete medium before plating.

### 2.5. Cytotoxicity Assays

MTT assays were used to probe cellular viability. RAW264.7 cells were seeded at a density of 5000 cells $well^{-1}$ (100 μL total volume $well^{-1}$) in 96-well assay plates. After 24 h incubation, the as-prepared peptide-AuNCs and peptide-AuNCs-CpG conjugates, at the indicated concentrations, were added for further incubation of 48 h. To determine toxicity, 10 μL of MTT solution (BBI) was added to each well of the micro titer plate and the plate was incubated in the $CO_2$incubator for an additional 4 h. Then the cells were lysed by the addition of 100 μL of DMSO. Absorbance values of formazan were determined with Bio-Rad model-680 microplate reader at 490 nm (corrected for background absorbance at 630 nm). Three replicates were done for each treatment group.

### 2.6. Fluorescent Microscopic Imaging

For the cell imaging test, the concentration of RAW264.7 cel was fixed at a density of $10^5$ cells $well^{-1}$in 24-well assay plates Then the FITC-labeled CpG ODNs, the peptide-AuNCs-CpG conjugates and the peptide-AuNCs-CpG conjugates with FITC-modified CpG ODNs were separately added to the cells and incubated at37°C for 4 h. The cells were then washed several times with PBS. Finally the imagings were captured using a Olympus BX-51 optical equipped with a CCD camera.

### 2.7. Cytokine Assays

RAW264.7 cells were seeded on 6-well culture plates at a density of $5 \times 10^5$ cells $well^{-1}$. After 24 h incubation, cells were washed with 0.5 mL PBS before treatment with indicated conditions (OVA peptide (1μg $mL^{-1}$), OVA peptide (1μg $mL^{-1}$)+ CpG ODNs (1.5μg $mL^{-1}$), peptide-AuNCs (containing 1 μg mL OVA peptide), peptide-AuNCs(containing 1 μg $mL^{-1}$ OVA peptide) + CpG ODNs (1.5μg $mL^{-1}$), or the peptide-AuNCs-CpG conjugates (containing 1 μg $mL^{-1}$ OVA peptide)) for8 h (TNF-α) or 24 h (IL-6). The supernatants were collected and stored at –80°C until use. The levels of TNF-α and IL-6 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using antibody pairs specific to these cytokines following protocols recommended by the manufacturer.

Splenocytes were isolated from the spleen of mice. Then, splenocytes were seeded on 6-well culture plates at a density of $5 \times 10^5$ cells $well^{-1}$, and treated with indicated conditions (OVA peptide (1μg $mL^{-1}$), OVA peptide (1μg $mL^{-1}$)+ CpG ODNs (1.5μg $mL^{-1}$), peptide-AuNCs (containing 1 μg $mL^{-1}$ OVA peptide), peptide-AuNCs (containing 1 μg $mL^{-1}$ OVA peptide) CpG ODNs (1.5μg $mL^{-1}$), or the peptide-AuNCs-CpG conjugates (containing 1 μg $mL^{-1}$ OVA peptide)) for 48 h (IFN-γ).The supernatants were collected and stored at –80°C until use. The levels of IFN-γ in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using antibody pairs specific to these cytokines following protocols recommended by the manufacturer.

### 2.8. Immunogenicity Study

Balb/C mice of 4–6 weeks were purchased from Medical Experimental Animal Center of Jilin University (Changchun, China). Female Balb/C mice were divided into different groups with four mice in each group. Mice were immunized with various equivalent dose samples through subcutaneous injection. 100 μL of peptide-AuNCs-CpG solution (containing 100 μg $mL^{-1}$ OVA peptide) was injected at each time point. The molar concentration of peptide(100 μg $mL^{-1}$ OVA peptide, 100 μL), peptide-AuNCs (containing 100 μg $mL^{-1}$ OVA peptide, 100 μL), and peptide-AuNCs (containing 100 μg $mL^{-1}$ OVA peptide 100 μL)+ CpG ODNs (150 μg $mL^{-1}$, 100 μL)was the same as that of the peptide-AuNCs-CpG conjugates and the amount of CpG ODNs is 150 μg $mL^{-1}$ (100 μL) at each time point. The molar concentration of peptide was adopted to confirm the molar concentration of peptide-AuNCs, peptide-AuNCs+ CpG ODNs and the peptide-AuNCs-CpG conjugates. Mice were injected on

days 0, 14, and 28, and sera were collected on day 42. All animal procedures were in accord with the guidelines of the Institutional Animal Care and Use Committee.

### 2.9. Titer Measurements

Micro titer plates (Nunc, Roskilde, Denmark) were coated with OVA by incubation of 100 µL 20 µg $mL^{-1}$ OVA in coating buffer (PBS, pH 7.2) for 24 h at 4 °C. To reduce non-specific binding, wells were blocked with 200 µL 1 w/v% BSA in PBST (0.05%Tween-20) for 1 h at 37 °C. After extensive washing with PBST, 20 dilutions of serum were added. After incubation for 1.5 h at 37 °C and washed three times, the second antibody of Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in goat was diluted to 1/2000and incubated (100 µL per well) for 1 h at 37°C. After washed four times, 0.4 mg $mL^{-1}$ o-phenylenediamine (4 mg o-phenylenediamine was dissolved in a buffer containing 4.86 mL 0.1 M critic acid and 5.14 mL 0.2M $Na_2HPO_4$) with 30% $H_2O_2$ (0.5 µL $mL^{-1}$) was added to wells (100 µL per well) as substrate. After 30 min incubation at room temperature, the color development was stopped by adding 50 µL of Stop Solution (2 M $H_2SO_4$) and optical absorption was measured at 490 nm.

## 3. Results and discussion

The detailed synthetic procedures were described in the Experimental Section. In brief, upon adding Au (III) ions to the aqueous peptide CCYSIINFEKL and thiolated CpG solution, Au ions could interact with the thiol groups of the peptide and thiolated CpG. The reduction ability of peptide was then activated by adjusting the reaction pH to ~12; the Au(III) ions then underwent progressive reduction to form AuNCs in situ (peptide-AuNCs-CpG conjugates).Without adding CpG ODNs, the same procedure gave the control (peptide-AuNCs).[62, 63]The optical properties (Fig.1a, S1) and fluorescence characteristics (Fig. 1a) of peptide-AuNCs-CpG conjugates were firstly investigated. The as-prepared peptide-AuNCs-CpG conjugates showed intense red fluorescence, with an emission peak at 600 nm. Fig. 1b showed the transmission electron microscopy (TEM) image of the peptide-AuNCs-CpG conjugates. The average diameter of AuNCs was estimated to be 1.17 nm. We then identified the triple ratio of Au, peptide and CpG molecules in the nanoclusters. After removing the unreacted reagents by dialysis using a dialysis tube,[64] ICP-MS was performed to quantify the amount of Au and DNA in the peptide-AuNCs-CpG conjugates. On the basis of the ICP-MS results (Fig. S2), we estimated that the triple ratio of Au to peptide to CpG was 49:6:2. Atomic force microscopy (AFM) was also carried out to characterize the morphology of the peptide-AuNCs-CpG conjugates (Fig. S3).The results showed that the heights of these AuNCs were approximately 1.3 nm, which were very close to their diameters observed by TEM. Zeta potential measurements (Fig. 1c) revealed that both peptide-AuNCs-CpG and peptide-AuNCs have negative surface charges (-17.57 mV and -15.34 mV, respectively). In the following, the energy-dispersive X-ray (EDS) measurement (Fig. 1d) clearly affirmed the primarily Au signals, further suggesting the formation of the AuNCs. X-ray photon spectroscopy (XPS) analysis (Fig. S4), gel electrophoresis (Fig. S5) a thermogravimetric analysis (TGA) (Fig. S6) were also carrie out to help verify the formation of the peptide-AuNCs-Cp conjugates. As high stability was imperative fo bioapplications,[65] we thus further interrogated the biologic stability of AuNCs. Interestingly, the emission of AuNCs was not perturbed over a broad pH range of 1–12 (Fig.S7), whic undoubtedly confirmed the robust fluorescence stability o AuNCs in different acidic/basic media. After that, the stabilit of the peptide-AuNCs-CpG against nuclease degradation was monitored in biological media. We incubated the peptide-AuNCs-CpG with 50% fetal bovineserum (FBS).[66, 67] FBS, which contains a mixture of nucleases and proteins,[68, 69] has bee widely employed to represent biological conditions fo investigating the stability of biomolecules[70] an nanomateirals.[71] The results presented in Fig. S8 and S exhibited good stability, clearly indicating the g conformational stability of the peptide-AuNCs-CpG.

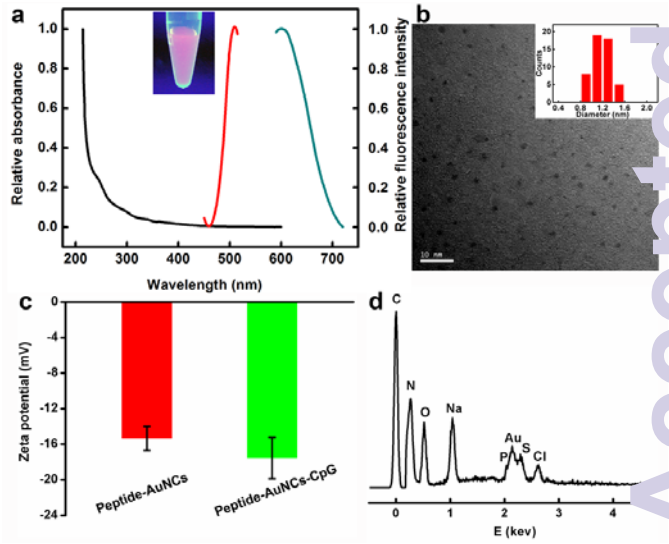



**Fig. 1** (a) The optical absorption spectrum, excitation and emission fluorescence spectra of the as-prepared peptide-AuNCs-CpG conjugates. Th insert photograph displays the luminescence of the AuNCs under a UV lamp. (b) TEM image of the peptide-AuNCs-CpG conjugates. Scale bar: 10 nm. Insert: size distribution histogram of the peptide-AuNCs-CpG conjugate The total number of clusters counted for the histogram was 50. (c) Zeta potentials of peptide-AuNCs and peptide-AuNCs-CpG conjugates. (d) ED spectrum of the as-synthesized peptide-AuNCs-CpG conjugates.

Before the application of the peptide-AuNCs-CpG conjugate in cells or tissues, the standard methyl thiazolyltetrazoliu (MTT) assay was carried out to evaluate the potentia cytotoxicity of the nanoplatform (Fig. 2a), which measured th mitochondrial activity of viable cells.[72]The viability o RAW264.7 cells was measured in the presence of the peptide AuNCs and peptide-AuNCs-CpG at various concentrations(2 100 µg $mL^{-1}$). The results revealed that the peptide-AuNCs and peptide-AuNCs-CpG showed no apparent cytotoxic effects o the RAW264.7 cell lines even at the highest concentration

tested (100 μg $mL^{-1}$), revealing that these AuNCs had high biocompatibility. Encouraged by the low cytotoxicity and excellent fluorescent properties of the peptide-AuNCs-CpG conjugates, we then made attempts to assess the potential of the peptide-AuNCs-CpG for cellular imaging(Fig. 3, S10), which served an indication of the cell uptake efficiency. Fig. 3showed the photoluminescence images of RAW264.7 cells after incubation with the FITC-labeled CpG ODNs, the peptide-AuNCs-CpG or the peptide-AuNCs-CpG with FITC-modified CpG ODNs. The RAW264.7 cells incubated with the FITC-labeled CpG ODNs alone showed negligible fluorescence signal (Fig. 3a), which was consistent with the facts that naked ODNs had difficulty passing through the cytoplasmic membrane and were also prone to degradation by nucleases.[73]Remarkably, bright red fluorescence was observed for cells treated with peptide-AuNCs-CpG (Fig. 3b), strongly demonstrating the effectiveness of using AuNCs for imaging applications. Overlays of the bright-field images and luminescence imaging demonstrated that the luminescence was evident in the intracellular region, indicating that AuNCs could facilitate the intracellular delivery of the peptide-AuNCs-CpG. Moreover, the results shown in Fig. 3c further confirmed the co-localization of green and red fluorescence signals in RAW264.7 cells after incubation with the peptide-AuNCs-CpG with FITC-modified CpG ODNs. This phenomenon indicated that both the peptide antigens and CpG ODNs could be presented into the same TLR9-positive cells, which might result in higher immune activity as compared to the free peptides or CpG ODNs alone. All these results demonstrated that the AuNCs could perform the bioimaging assignment and facilitate simultaneous co-delivery of the peptide antigens and adjuvant CpG ODNs to the same APCs, which could promote cross-presentation and help elicit a stronger immune response.[74-76]

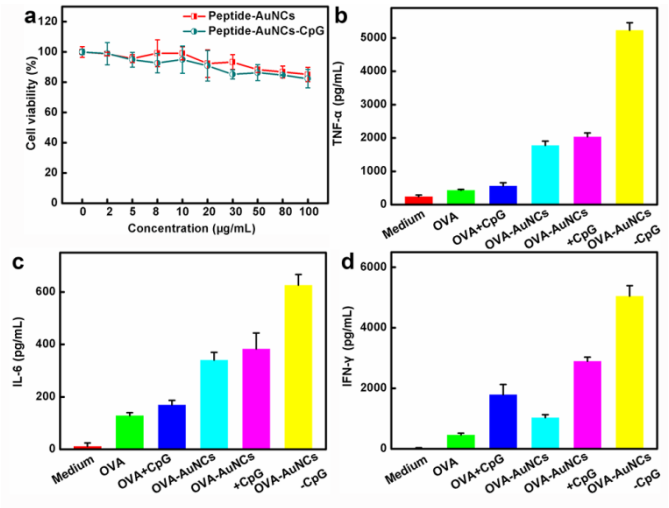



**Fig. 2** (a) Cytotoxicity testing results of the peptide-AuNCs and the peptide-AuNCs-CpG conjugates against RAW264.7 cells by MTT assays. (b, c) Cytokine release from RAW264.7 cells stimulated by the peptide-AuNCs-CpG conjugates. Comparison of (b) TNF-α and (c) IL-6 release stimulated by OVA peptide, OVA peptide+ CpG ODNs, peptide-AuNCs, peptide-AuNCs+CpG ODNs, and the peptide-AuNCs-CpG conjugates. Error bars represent standard deviation of three independent measurements. (d) IFN-γ production in the splenocyte assay. The splenocytes were isolated from the spleen of mice. Splenocytes were stimulated by OVA peptide, OVA peptide + CpG ODNs, peptide-AuNCs, peptide-AuNCs + CpG ODNs, and the peptide-AuNCs-CpG conjugates, and IFN-γ concentration in day 3supernates was determined by cytokine ELISA.

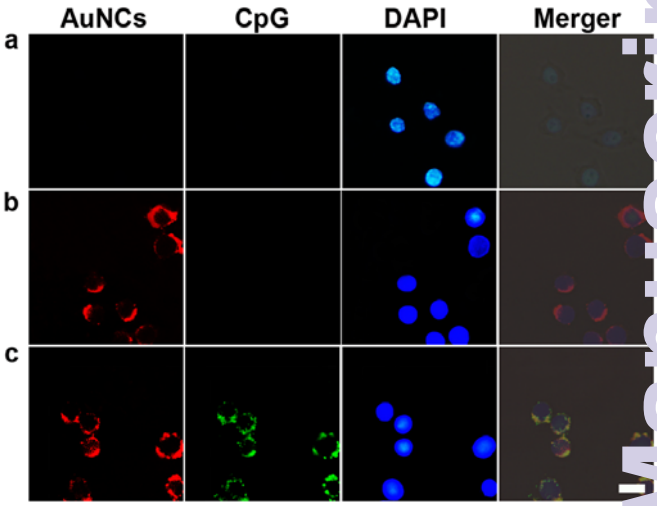



**Fig. 3**Fluorescence images ofRAW264.7 cells treated with (a) FITC-labeled CpG ODNs (2 μM), (b) the peptide-AuNCs-CpG conjugates (50 μg $mL^{-1}$) or (c) the peptide-AuNCs-CpG conjugates with FITC-modified CpG ODNs (50 μg $mL^{-1}$). Scale bar: 20 μm.

Encouraged by these attractive advantages, we then investigated the immunostimulatory effect of the peptide-AuNCs-CpG conjugates. Since production of proinflammatory cytokines played a pivotal role in eliciting innate immune responses as well as in priming and coordinating adaptive immune responses,[77] we hence incubated these nanovaccines with macrophage RAW264.7 cells and examined their immunostimulatory activities by measuring the release of cytokines using the enzyme-linked immunosorbent assay (ELISA). Samples were cultured withRAW264.7 cells and the levels of the secreted TNF-α and IL-6 was recorded as a measure of immunostimulation. As can be seen in Fig. 2b, peptide-AuNCs dramatically induced the production of TNF-α, excelling that of the free OVA peptide by more than 4 times. We used circular dichroism (CD) to investigate the effect of AuNCs on peptide secondary structure. As shown in Fig. S11, no obvious change of the CD spectrum was observed for peptide-AuNCs as compared to the free peptide. The result suggested that the synthesis of AuNCs in OVA peptide molecules had little effect on the structure of the peptide scaffolds, which provided the opportunity to preserve the bioactivity of the natural OVA peptide.[50] Besides, compared with OVA peptide, the peptide-AuNCs offered additional benefits, such as enhanced cellular uptake efficiency,[40, 78] increased stability and high resistance to nuclease degradation,[79] which thus could significantly increase the immunostimulatory efficacy. Notably, when comparing the peptide-AuNCs-CpG conjugates to a mere mixture of peptide-AuNCs and free CpG ODNs, the huge increase was noted on the level of TNF-α, clearly reflecting that concomitant delivery of antigens and CpG ODNs to the same APCs was indeed beneficial for the enhanced cellular immunity. Similarly,

compared with the OVA and CpG control, the level of the other cytokineIL-6 (Fig. 2c) induced by the peptide-AuNCs-CpG conjugates was also greatly increased, demonstrating the feasibility of using the peptide-AuNCs-CpG conjugates as the potential nanovaccines. It was exciting to see that, without the need of vaccine carriers, the peptide-AuNCs-CpG conjugates could act as self-vaccines to help in the generation of enhanced immunostimulatory activity.

In addition, the peptide-AuNCs-CpG conjugates could also induce the expression of the splenocyte cytokine. IFN-γ plays a critical role in many immunoregulatory processes, including the activation of mononuclear phagocytes and promoting the differentiation of fully cytotoxic T lymphocytes(CTLs) from $CD8^+$Tcell precursors,[80]which make it a central cytokine that orchestrates T cell-mediated immunity against tumor cells.[81]In order to measure the level of IFN-γ production, splenocytes isolated from the spleen of mice were stimulated by the vaccine samples, and tissue culture supernates were collected at day 3 for cytokine ELISA assays.[82]As shown in Fig. 2d, the peptide-AuNCs-CpG conjugates induced the highest levels of cytokine IFN-γ release. By comparison, OVA peptide plus CpG and peptide-AuNCs vaccination resulted in IFN-γ production approximately 35% and 20% of this amount, respectively. In addition, the group immunized with peptide-AuNCs was more effective at generating IFN-γ than OVA peptide alone. These results were consistent with previous studies that, through the combined delivery of OVA peptide and CpG into the same APCs, the immunogenicity of the peptide-AuNCs-CpG was greatly enhanced.[20, 60, 80]Taken together, these data suggested that the peptide-AuNCs-CpG conjugates were able to trigger a strong intracellular signaling leading to the activation of the production of cytokines, such as TNF-α and IL-6, induce the differentiation of naive T cells into $CD8^+$ T cells, as seen by the higher INF-γ secretion.[81]

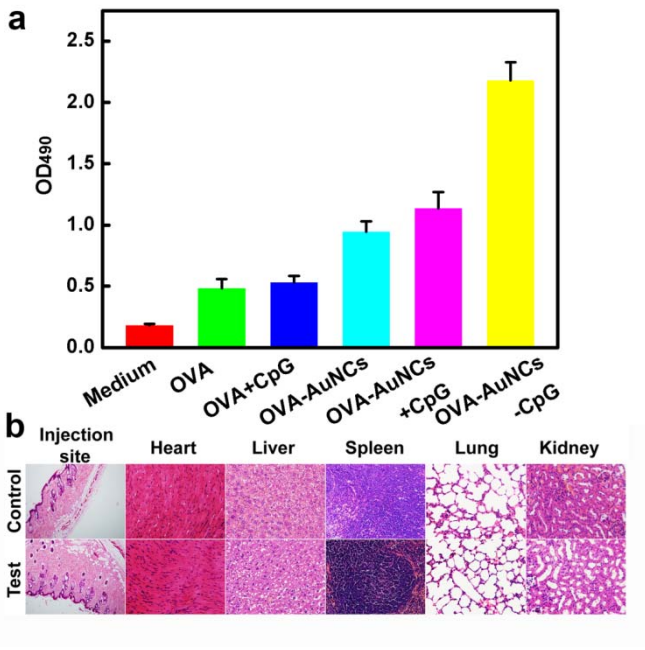



**Fig. 4**(a) ELISA results of anti-OVA IgG titers elicited by different stimulus. (b) Histopathological studies of tissue organs from a mouse injected with the peptide-AuNCs-CpG conjugates (top panel) and a non-immunized control animal (bottom panel). These organs are stained with haematoxylin and eosin (H&E) and observed under a light microscope. Tissues were harvested from injection site, heart, spleen, liver, lung, as well as kidney.

To go one step further in the application of the peptide-AuNCs-CpG conjugates in immunostimulation, we further tested whether the nanoparticles complexes could also induce antigen-specific immune responses in vivo. Balb/c mice were vaccinated subcutaneously with formulations described in the experimental section and the anti-OVA specific total serum IgG titres were measured by immunoglobulin ELISA. As shown in Fig. 4a, the amount of anti-OVA IgG vaccinated with peptide-AuNCs was about 2 fold more than that of the free peptide, demonstrating that, compared with OVA peptide, the immunization with the peptide-AuNCs substantially enhanced the OVA-specific IgG antibody titers. We reasoned the enhanced immunostimulatory effects were due to the fact that more OVA peptides were protected from degradation and could be efficiently internalized into the APCs. Notably, vaccination with the peptide-AuNCs-CpG conjugates resulted in much higher number of anti-OVA IgG compared to that of peptide-AuNCs mixed with free CpG ODNs. This result implied that co-administration of antigen with adjuvants into the same APCs generated better immune responses than that of antigen or CpG alone.[83-85]Next, the toxicity of the peptide-AuNCs-CpG conjugates was further evaluated by analyzing the tissues from the organs harvested from two mice for each group at the time of necropsy. Tissues including injection site, heart, liver, spleen, lung, and kidney were collected and examined (Fig. 4b).[61]Hemotoxylin and eosin staining showed that there were no discernible morphological changes in the tissue at the site of injection in mice treated with the peptide-AuNCs-CpG conjugates as compared with the unimmunized controls. In addition, no inflammation, cell necrosis and apoptosis were observed in heart, liver, spleen, lung and kidney of mice administrated with the peptide-AuNCs-CpG conjugates, similar to the untreated control group. These data indicated that the self-vaccines peptide-AuNCs-CpG conjugates presented high biocompatibility and showed more promising for further immunotherapy.

## 4. Conclusion

In summary, we developed a facile method to produce the peptide-AuNCs-CpG conjugates using the bifunctional peptide antigens and thiolated adjuvant CpG ODNs. The synthesized AuNCs exhibit red emission, and are able to act as smart self-vaccines to assist in generation of high immunostimulatory activity. The introduction of the peptide-AuNCs-CpG conjugates brings about several unprecedented advantages. Firstly, the AuNCs are simple to synthesize, commercially affordable, and exhibit no cytotoxicity, which make them a good candidate for biomedical applications. Secondly, the peptide-AuNCs-CpG conjugates can simultaneously deliver

peptide antigens and CpG ODNs into the same APCs, thus can promote cross-presentation and help elicit the strong immunostimulatory effects both in vitro and in vivo, especially mount strong $CD8^+$ CTL responses. Furthermore, the peptide-AuNCs-CpG conjugates with fascinating optical properties can also simultaneously serve as an imaging agent without any further modification. Collectively, our studies provide strong evidence that the peptide-AuNCs-CpG conjugates can be utilized as efficient immunostimulatory agents that are able to prevent and/or treat a variety of ailments.

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