Surface-Engineered Gold Nanorods: Promising DNA Vaccine Adjuvant for HIV-1 Treatment

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S *Supporting Information*

ABSTRACT: With the intense international response to the AIDS pandemic, HIV vaccines have been extensively investigated but have failed due to issues of safety or efficacy in humans. Adjuvants for HIV/AIDS vaccines are under intense research but a rational design approach is still lacking. Nanomaterials represent an obvious opportunity in this field due to their unique physicochemical properties. Gold nanostructures are being actively studied as a promising and versatile platform for biomedical application. Herein, we report novel surface-engineered gold nanorods (NRs) used as promising DNA vaccine adjuvant for HIV treatment. We have exploited the effects of surface chemistry on the adjuvant activity of the gold nanorod by placing three kinds of molecules, that is, cetyltrimethylammonium bromide (CTAB), poly(diallydimethylammonium chloride) (PDDAC), and polyethyleneimine (PEI) on the surface of the nanorod. These PDDAC- or PEI-modified Au NRs can significantly promote cellular and humoral immunity as well as T cell proliferation through activating antigen-presenting cells if compared to naked HIV-1 Env plasmid DNA treatment in vivo. These findings have shed light on the rational design of low-toxic nanomaterials as a versatile platform for vaccine nanoadjuvants/delivery systems.

KEYWORDS: Gold nanorod, surface chemistry, adjuvant, DNA vaccine, HIV-1
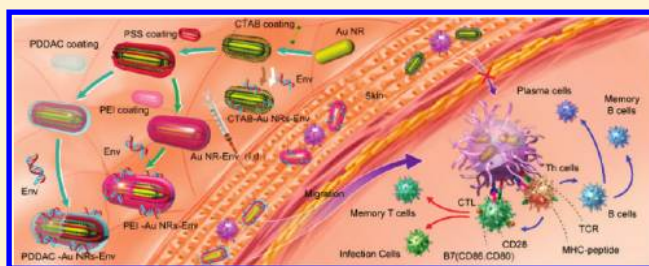
More than 60 million people worldwide have been reported to be infected with HIV-1, mostly in developing countries, and nearly half of them have died.[1] Meanwhile, more than 6500 new infections emerge daily.[2] HIV-1 has advanced from high-risk groups such as intravenous drug users to the general population in China.[3] HIV-1 has been one of the most catastrophic pandemics to confront mankind. Although vaccines are effective against many infectious diseases, HIV-1 vaccines have failed due to safety issues or lack of efficacy in humans despite extensive investigation. For instance, a very promising HIV-1 candidate vaccine from Merck, Ad5 trivalent vaccine, gave disappointing results in 2007.[4] In view of the potential risks associated with some traditional vaccines comprised of live-attenuated or killed bacteria or viruses, new generation vaccines such as protein- and DNA-based vaccines have emerged and attracted much attention.

Compared to protein vaccines, DNA vaccines can generate long-lived cellular immunity in addition to humoral immune response in both experimental systems and humans and can provide protective immunity in animal challenge models.[5−7]

Furthermore, DNA vaccines are safer, relative inexpensive for manufacture and storage, and thus have the potential for simultaneous immunization against multiple antigens or pathogens.[8] Therefore, DNA vaccine has become the focus of vaccine development. Despite the various advantages, the immunogenicity of DNA vaccine has been poor for human and nonhuman primates[8] due to poor uptake by antigen-presenting cells (APCs) and DNA degradation by nuclease.[9] To solve these problems and improve the immunogenicity of DNA vaccines, it is mandatory to develop a safe and effective vaccine adjuvant/delivery system.

Until now, aluminum compounds and MF59 have been the two dominant adjuvants that can be used safely in humans. However, aluminum compounds mainly generate a humoral immune response, and the cellular immune response generated by MF59 is not active enough.[10] Thus, these two adjuvants are
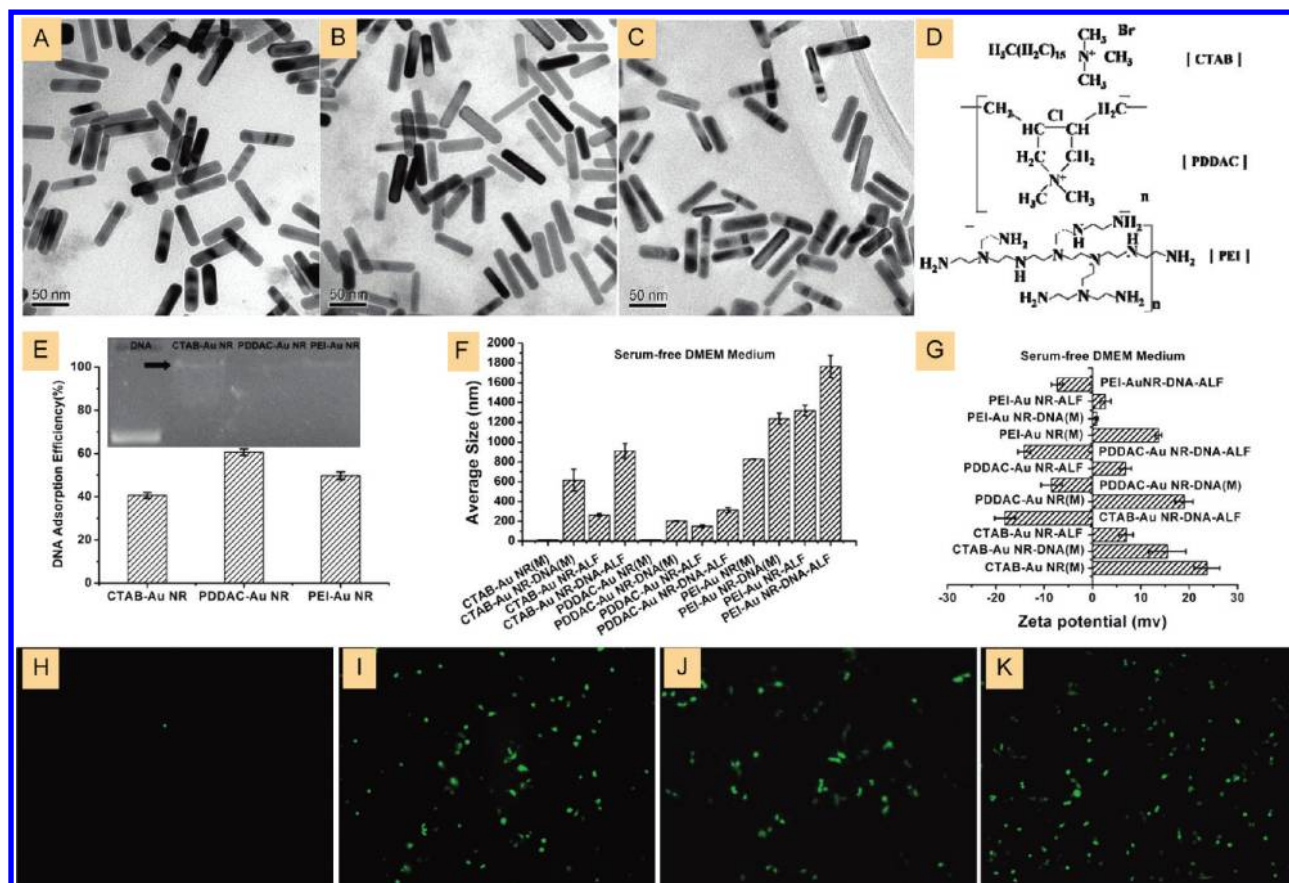
Figure 1. The characterization, DNA adsorption capability, and cell transfection activity of Au NRs. TEM images of CTAB-Au NRs (A), PDDAC-Au NRs (B), and PEI-Au NRs (C). The chemical structures of CTAB, PDDAC ($n = 660-1320$) and PEI ($n = 52$) (D). (E) Comparison of DNA adsorption by Au NRs. Among three kinds of Au NRs, PDDAC-Au NR has largest DNA adsorption capability. As a result of the higher density of Au NR-DNA complex, the location of DNA band lagged compared to naked DNA (indicated by black arrow). (F,G) The DLS characterization for Au NRs and the Au NR-DNA complex within serum-free DMEM medium or artificial lysosomal fluid (ALF). The increased size distribution indicates that CTAB-Au NR and PEI-Au NR had poor stability when added into serum-free medium containing DNA and ALF. Comparison of cell transfection capability by CTAB- (H), PDDAC- (I), and PEI-Au NR (J). PEI serves as a positive control (K). HEK293 cells were treated with the Au NR-DNA complex for 3 h. Photos were taken under an Olympus inverted fluorescence microscope at 48 h post transfection. The magnification is 16 × 10.

not ideal for eliminating endogenous pathogens like HIV-1. Because of the advantages in safety over viral carriers, nonviral vehicles are currently being extensively investigated in the development of vaccine carriers and adjuvants. As nonviral vehicles, nanomaterials have advantages over bulk materials of the same compositions due to their unique structure and properties, such as smaller size, large surface area-to-volume ratio, ease of preparation, and potential for modification, to name a few.

As carriers, nanomaterials have been found to enhance the cellular uptake of DNA, lengthen the circulation time, target specific cells and regulate immune response.[11,12] Several kinds of nanomaterials have been investigated as vaccine carriers and/or adjuvants.[13–17] For instance, it was found that PLGA nanoparticle could alter the type of immune responses elicited by the Th2-biased antigen into Th1 immune responses.[13] Uto and colleagues reported the adjuvant activity of poly(γ-PGA) nanoparticle and found that it induced innate and adaptive immunity via TLR4 and MyD88 signaling pathway.[14,18] However, systematic investigation is lacking, and rational selection of adjuvants remains rather empirical. Meanwhile, there were no nanomaterials (smaller than 100 nm) exploited as adjuvant for HIV-1 DNA vaccine. Recently, gold nanorods

(Au NRs) have demonstrated promising potential in bioimaging, immunoassays, thermal therapy for tumors, and drug delivery including gene delivery.[19–24] One report[21] showed that gold nanorods could be used as vehicles to enhance the cellular uptake of an ssRNA immune activator to inhibit the pandemic H1N1 influenza viral replication in vitro. The interactions of nanoparticles with biological systems play key roles in executing their biomedical functions and in toxicity. The toxicological consequences of nanoparticles in vivo and cellular responses are highly related to their properties like size and shape, composition, charge, and surface chemistry. Our previous studies have showed that the cellular uptake and toxicity of gold nanorods depended on the surface chemistry and aspect ratio.[25,26] Recent results demonstrated that poly(diallyldimethyl ammonium chloride) (PDDAC)-coated Au NRs showed low toxicity and better internalization[27] and serum protein-coated Au NRs could selectively target to the mitochondria of cancer cells and then kill them.[28] Accumulating evidence demonstrated that the behavior and bioactivity of nanomaterials determined by multiple parameters. For instance, a direct comparison of PLG microparticles and nanoparticles was investigated by several groups but results were not consistent.[29,30] It should be noted that nanoparticle character-

ization has been a rate-limiting step that hinders the development and prospective uses of some promising nano-materials like hybrid inorganic–organic core–shell nano-particles. To solve this problem, Stellacci and his colleagues developed a method of general applicability to determine the size distribution of nanoparticles.[31] Meanwhile, they found that the distribution manner of surface composition regulated the pathway of gold nanoparticle entry into cells.[32] Binding of blood proteins can reduce the cytotoxicity of nanomateri-als.[33,34] Although great progress has been made in improving the in vitro transfection ability of nanomaterials,[35–40] their adjuvant activity in humans has yet to be demonstrated due to the complexity of the in vivo experimental system and the differences between animals and humans.

In this study, we have investigated the in vivo adjuvant activity of Au NRs as possible vehicles for gene delivery with a specific focus on the role of the surface chemistry of the rods to achieve the rational safe design. Two cationic molecules were selected to modify the surface of the Au NRs: PDDAC and polyethyleneimine (PEI). PDDAC-Au NRs have high stability under various conditions and no obvious cytotoxicity, whereas PEI-Au NRs may have additional advantages such as the proton sponge effect. The original rod-forming surfactants, CTAB-Au NRs, and commercial transfection reagent-PEI were employed as controls. HIV-1 Env plasmid DNA was chosen as the antigen. In vitro results demonstrated that except for CTAB-Au NRs, PDDAC- and PEI-Au NRs had good transfection capability. Both cellular and humoral immunity were enhanced significantly when mice were immunized with PDDAC-Au NR-Env and PEI-Au NR-Env. For the PDDAC-Au NR-Env group, the type of immune response was Th2-biased. PDDAC-Au NRs and PEI-Au NRs could promote the maturation of dendritic cells (DCs) but CTAB-Au NRs could not. DC maturation may be the mechanism for the positive effects of PDDAC-Au NRs and PEI-Au NRs as vaccine adjuvant/delivery systems. This is the first report on Au NRs used as DNA vaccine adjuvants. We expect that our study provides an insight into the rational design of nanomaterials as adjuvant/delivery systems.

Enhanced Transfection Capability Dependent on Surface Chemistry of Gold Nanorods. Original CTAB-Au NRs with an average aspect ratio of 4 were synthesized via the seed-mediated growth method.[41,42] PDDAC- and PEI-Au NRs were obtained via layer by layer electrostatic assembly.[43] The Au NRs have similar sizes (15 nm × 60 nm, Figure 1A–C). Instead of the HIV-1 Env plasmid, plasmid-enhanced green fluorescent protein (pEGFP) was used to assess the trans-fection ability of Au NRs and was bound to the three kinds of Au NRs via electrostatic interaction. PDDAC- and PEI-Au NRs had transfection capability comparable to that of the commercial transfection agent-PEI (branched, MW = 25 kD) when observed at 48 h post-transfection (Figure 1I–K). In contrast, CTAB-Au NRs could barely transfect HEK293 cells at all (Figure 1H). At 24 h post-transfection, few cells expressed the green fluorescent protein (GFP) for the PDDAC-Au NR-DNA treatment group, whereas a greater expression was observed for the PEI-Au NRs and PEI-positive group (Figure S1, Supporting Information). No expression was found for the CTAB-Au NRs treatment group at this time point (Figure S1, Supporting Information). We hypothesize that faster trans-fection responses for PEI-Au NRs and PEI may result from the "proton sponge" effect of PEI molecules.

Different DNA Adsorption Capability and Stability within the Biological Environment of Au NRs with

Different Surface Modifiers. For effective transfection, the first factor to be considered is DNA adsorption efficiency onto the Au NRs. The DNA adsorption efficiency for CTAB-Au NRs, PDDAC-Au NRs, and PEI-Au NRs was 41, 61, and 50%, respectively, which is in agreement with the results from agarose electrophoresis (Figure 1E). These values translate to DNA-loading capacities of 3.4×10^{-3}, 5.1×10^{-3}, and 4.1×10^{-3} μM/mM, respectively. There was no visible DNA band from the supernatants of the PDDAC-Au NR-DNA or PEI-Au NR-DNA complexes. The DNA band intensity for the supernatant of the CTAB-Au NR-DNA complex was weaker than the DNA control.

The changes in the gold nanorods after adsorbing DNA were checked from UV–vis-NIR absorption spectra. For the CTAB-Au NRs, the longitudinal surface plasmon resonance (LSPR) absorption band broadened a little after adsorbing DNA, indicating a slight aggregation of the rods (Figure S2A, Supporting Information). Dynamic light scattering (DLS) experiments verified the formation of aggregates. The effective size increased from 12.9 ± 0.1 to 617.1 ± 109.9 nm after mixing with serum-free DMEM containing pEGFP (Figure 1F, Figure S2C, Supporting Information), while the zeta potential decreased from 23.7 ± 2.7 to 15.5 ± 3.9 mV (Figure 1G, Figure S2E, Supporting Information). After it was added to the artificial lysosomal fluid (ALF), the size of the CTAB-Au NR-DNA complex was further increased to 910.4 ± 75.1 nm. For PDDAC-Au NRs, the LSPR band showed only a small red shift of 10 nm with a slight broadening. The size measured by DLS increased from 13.3 ± 0.2 to 202.8 ± 4.1 nm (Figure 1F, Figure S2C, Supporting Information), while the zeta potential decreased from 18.9 ± 1.9 to -8.5 ± 2.2 mV (Figure 1G, Figure S2E, Supporting Information). The reversal of the zeta potential indicates more DNA adsorption, which is in agreement with the results of Figure 1G. Within the environment of the ALF, the size of the PDDAC-Au NR-DNA complex increased to 312.8 ± 24.9 nm (Figure 1F, Figure S2C, Supporting Information). Finally, PEI-Au NR aggregated in serum-free medium, while after it was mixed with serum-free medium containing plasmid DNA (pEGFP), the effective size increased from 828.9 ± 4.6 to 1237 nm ± 55 nm (Figure 1F, Figure S2C, Supporting Information). It seems that the aggregation status of the nanorods did not affect the transfection of PEI-coated Au NR. Note that due to the different chemical structures of the surface coatings (Figure 1D), the nanorods differed in their aggregation after adsorbing DNA, which may influence their ability to enter cells (see below).

Internalization and Cellular Trafficking within Host Cells with Minimal Cytotoxicity. Intracellular trafficking is crucial for gene delivery. Dynamic localization was employed to check the intracellular location and the delivery processes of the Au NR-DNA complex by monitoring the attached DNA. To monitor Au NR-pEGFP complex in cells, the red fluorescent probe Cy3-dCTP was used to label pEGFP through the nick translation method. The lysosomes and mitochondria were labeled with the tracker probes LysoTrackerGreen DND-26 and MitoTrackerGreen FM, respectively. Nuclei were stained with Hoechst 33342. The location of the Au NRs-DNA complex or DNA could be clearly seen through the colocalization of Au NR-DNA or DNA with other organelles. PEI was used as a positive control.

For the PDDAC-Au NR-DNA and PEI-Au NR-DNA complexes, DNA could transport into the nucleus even at 2.5
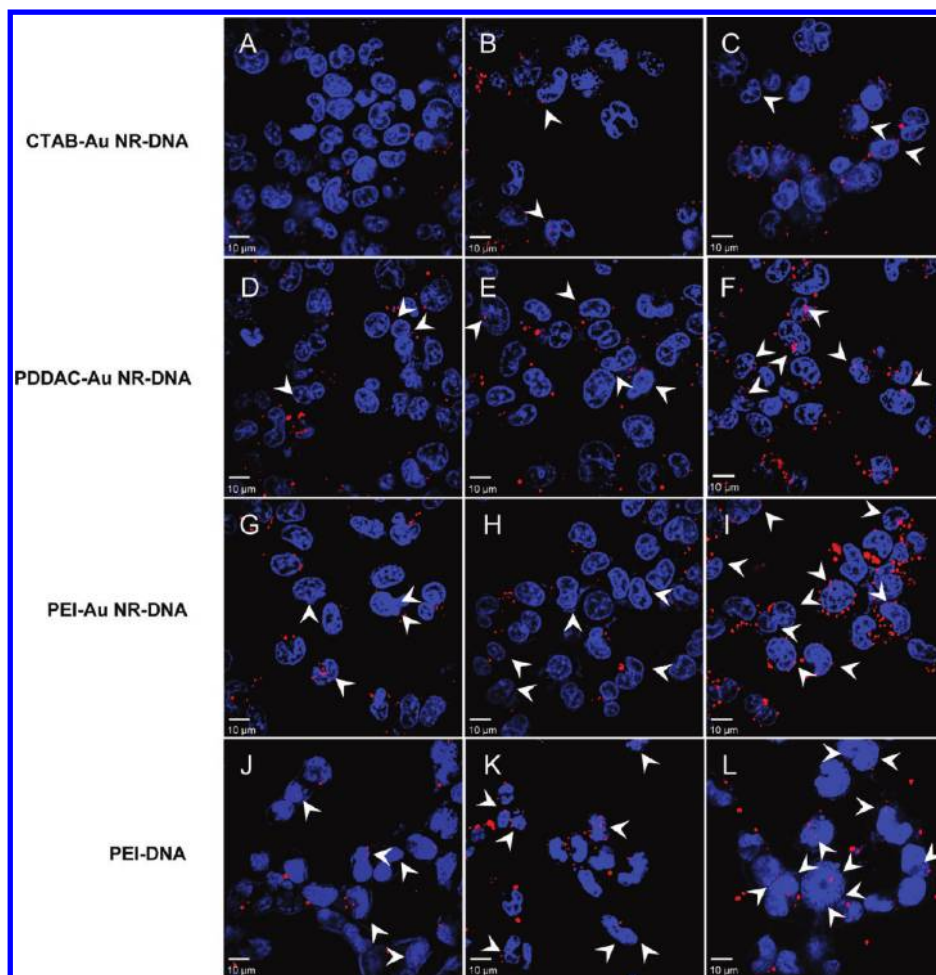
Figure 2. Co-localization of DNA and nuclei at different time points for Au NR-DNA and PEI-DNA complexes. (A−C) CTAB-Au NR-DNA at 2.5, 17 and 22 h. (D−F) PDDAC-Au NR-DNA at 2.5, 5, and 22 h. (G−I) PEI-Au NR-DNA at 2.5, 5, and 15 h. (J-L) PEI-DNA at 2.5, 6, and 10 h. These results were confirmed at different fields under CLSM. Results show that DNA could transport into the nucleus at an early stage post-treatment (e.g., 2.5 h) when cells were treated with PDDAC-Au NR-DNA, PEI-Au NR-DNA and PEI-DNA complexes for 3 h. But there were few colocalization between CTAB-Au NR-DNA complex and nuclei even at 22 h post treatment.

h post-transfection, and more colocalization was observed at longer time points (Figure 2D−I). PEI-DNA complexes showed a similar trend (Figure 2J−L). In contrast, for CTAB-Au NR-DNA complex only a small amount of colocalization of DNA with the nucleus was observed at 2.5 h post-transfection, and the colocalization did not show obvious increases even at 17 and 22 h (Figure 2A−C). All three kinds of Au NR-DNA complexes were localized in lysosomes at 2.5 h post-transfection (Figure S3A−C, Supporting Information). Most of the CTAB-Au NR-DNA complexes located in lysosomes with a few colocalizations with mitochondria at different time points (Figure S4A-4C, Supporting Information). No colocalizations of PDDAC-Au NR-DNA or PEI-Au NR-DNA with mitochondria were found at any time points (Figure S4D−I, Supporting Information), in agreement with our previous results.[27,28]

To find the localization of gold nanorods inside the cell, transmission electron microscopy (TEM) was used. The CTAB-Au NR-DNA complex was mainly located in endosomes or lysosomes, and sometimes the complex could be seen in mitochondria at different time points (Figure S5A, Supporting Information), consistent with fluorescence results. The PDDAC-Au NR-DNA and PEI-Au NRs-DNA complexes were only located in endosomes or lysosomes at all time

points (Figure S5B,C, Supporting Information). This evidence confirmed the results of the dynamic localization of the Au NR-DNA complex with confocal laser scanning microscopy (CLSM).

As the first barrier, efficient cellular internalization of the carrier-DNA complex is crucial for nonviral vehicles. We quantified the internalization of the Au NR-DNA complex into cells with inductively coupled plasma mass spectrometry (ICP-MS). After 3 h of incubation, the internalization of PDDAC-Au NRs-DNA was 1.5 times that of CTAB-Au NR-DNA and twice that of PEI-Au NR-DNA (Figure 3C). The smaller sizes of the PDDAC-Au NR-DNA complexes may be responsible for their higher internalization (Figure 1F, Figure S2C, Supporting Information).

Effective DNA Release Depends on the Surface Chemistry of Au NRs. Effective DNA release is another critical issue determining protein translation (e.g., GFP, protein antigens). The fluorescence of a dye adsorbed on Au NRs can be quenched. The fluorescence intensity of Cy3 labeled-DNA apparently decreased after DNA was mixed with Au NR as shown in Figure 4a. After the DNA is released from the Au NRs, its fluorescence can be recovered, thus providing a simple way to monitor the release. The fluorescence intensity of Cy3-DNA within HEK293 cells increased with time for the
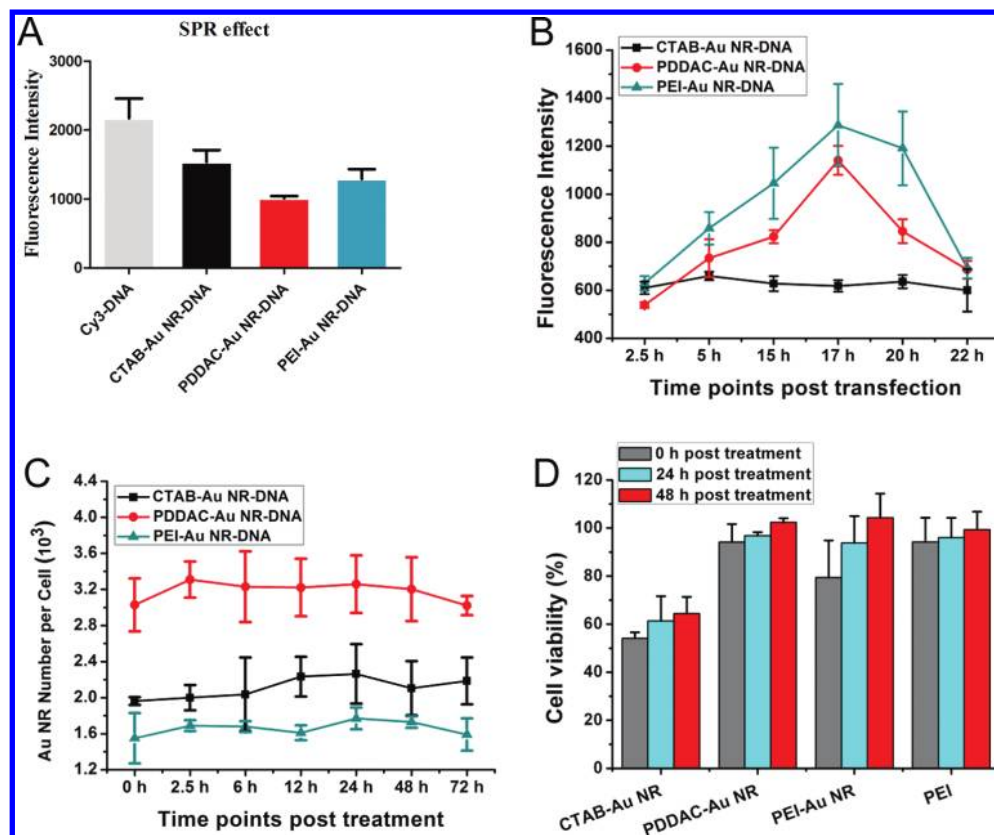
Figure 3. The evaluation of the SPR effect, the internalization quantity and cell viability after treatment with Au NRs or Au NR-DNA. (A) The SPR effect of Au NRs in vitro. The fluorescence intensity was determined after Cy3-DNA (0.8 μg/mL) was mixed with Au NR (100 μg/mL) at a volume ratio of 1:1. The fluorescence intensity decreased the most after Cy3-DNA was incubated with PDDAC-Au NR. The second largest decrease was PEI-Au NR, which means that less free Cy3-DNA remains for PDDAC- and PEI-Au NR-DNA mixtures. It is consistent with the result of DNA adsorption capability. (B) The intensity of Cy3-DNA within HEK293 cells through the software Volocity of CLSM at different time points after cells were treated with Au NRs-DNA for 3 h. The Cy3-DNA fluorescent intensity recovered within a longer time in the PDDAC- and PEI-Au NR treated groups, which indicates that Cy3-DNA could be released from the surface of the Au NRs except for CTAB-Au NR. (C) The internalization quantity determined by ICP-MS. The internalization amount of PDDAC-Au NR-DNA was 1.5 and 2 times than that of CTAB- and PEI-Au NR-DNA complexes, respectively. (D) The effects of Au NRs on cell viability only show that CTAB-Au NRs are toxic to cells. Cell viability was determined at 0, 24, and 48 h post-treatment after cells treated with Au NR-DNA complex for 3 h.

PDDAC-Au NR-DNA and PEI-Au NR-DNA treatment groups. However, no increase in fluorescence intensity with time was observed for the CTAB-Au NR-DNA group (Figure 3B).

How can DNA escape from the endosome/lysosome? The accepted mechanisms to explain DNA release from endosomes or lysosomes are endosomal membrane disruption and the proton sponge effect.[44] A typical example of the proton sponge effect is PEI, which can effectively buffer the pH of the endosomal compartment. This effect results in an increased influx of protons (and hence chloride ions and water), thus causing swelling and ultimately osmotic lysis. As for CTAB and PDDAC, instead of the "proton sponge effect", positive charges provided by quaternary ammonium groups endow them with the capability to disrupt the endosomal or lysomal membrane. Under this condition, pEGFP could escape from the endosome or lysosome and transport into the nucleus.

At first glance, the difference in transfection capability between CTAB-Au NRs and PDDAC-Au NRs might appear to come from the large aggregation size of the CTAB-Au NR-DNA complex (910 nm in ALF). However, the PEI-Au NRs possess an even larger aggregation size (1900 nm in ALF). One possible reason might be that the CTAB molecules can destroy DNA. A small decrease in the A275 nm/A221 nm ratio of DNA from CD spectra in the presence of CTAB-Au NRs may

suggest some changes in the plasmid DNA conformation, whereas no obvious changes were found for the other two Au NRs (Figure S6, Supporting Information). At the moment, the exact mechanism of the low transfection capability for CTAB-Au NRs is unclear and needs further investigation. Considering that they have the lowest internalization quantity and largest aggregation size among the three Au NRs, we believe that the high transfection efficiency of PEI-Au NRs results from the proton sponge effect. From these results, a potential mechanism can be drawn as shown in Figure S7, Supporting Information. After being internalized into cells, the Au NR-DNA complex is encapsulated into endosomes. For the CTAB-Au NR-DNA complex, it would stay first in the endosome and then for a longer time in the lysosome, where the DNA is destroyed by nucleases and acidic enzymes. Additionally, colocalization with mitochondria at different time points may produce a negative effect as mitochondria are crucial for all kinds of cell activations (including DNA expression). The cell viability evaluation also showed that in comparison with the other two Au NRs, CTAB-Au NR was more toxic to HEK293 cells (Figure 3D). For the PDDAC-Au NR-DNA and PEI-Au NR-DNA complexes, DNA can escape from endosomes and then translocate rapidly into the nucleus. Then, events such as green fluorescent protein translation occur. For antigen
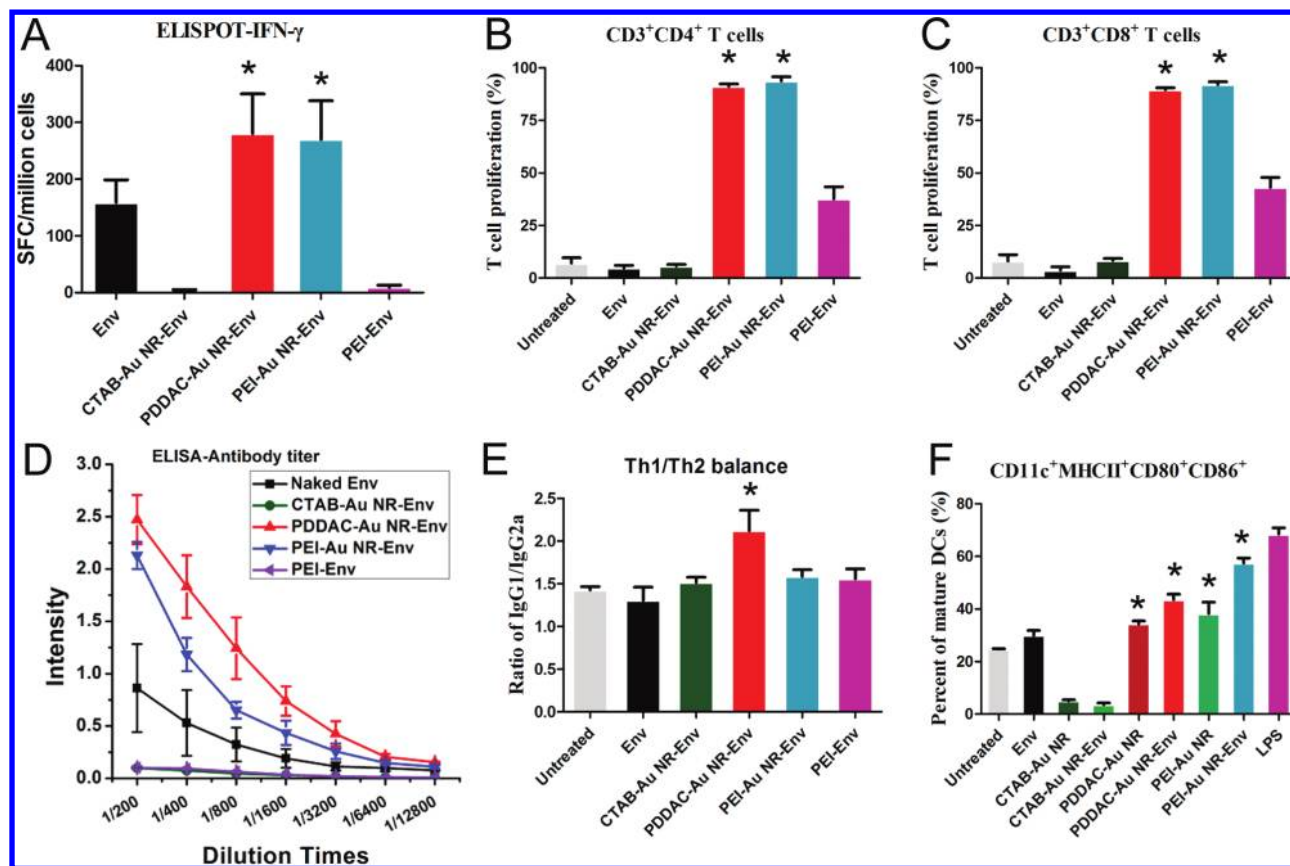
Figure 4. The effects of Au NRs on the immune response and dendritic cell maturation. (A) IFN-γ analyzed by ELISPOT. (B) CD3⁺CD4⁺ T cell proliferation. (C) CD3⁺CD8⁺ T cells proliferation. (D) The Env specific antibody titer measurement. (E) Determination of the type of immune response after mice immunized with Au NR-Env plasmid DNA complex. (F) The effect of Au NRs and the Au NR-Env complex on DC maturation. Results indicated that the IFN-γ secretion level, the antibody titers, and T cells proliferation capability were significantly enhanced after mice were immunized with the PDDAC- and PEI-Au NR-Env complexes. The type of immune response for the PDDAC-Au NR-Env treatment group was Th2-biased. Meanwhile, Both PDDAC and PEI-Au NRs together with their Env complex could promote DC maturation, which could play an important role in their enhanced vaccine adjuvant activity of Au NR with proper modification.

presenting cells (APCs), protein will transport into lysosomes and be processed. Finally, the antigen is presented in the form of an MHC-peptide complex that then activates the T and/or B cells. In vitro, PEI-coated Au NRs have the best transfection efficiency among the three Au NRs.

Promising Vaccine Adjuvant/Delivery System Based on Gold Nanorods. Three Au NRs with different surface coatings (CTAB-, PDDAC-, and PEI-) were examined to determine whether they could enhance the immunogenicity of Env plasmid DNA. When the PDDAC-Au NR-Env and PEI-Au NR-Env plasmid DNA groups were immunized with intradermal injection, the results of ELISPOT determination for IFN-γ, which is a typical representative for cellular immunity, showed that the IFN-γ secretion level was enhanced significantly compared with a naked Env plasmid DNA group (Figure 4A). However, CTAB-Au NR-Env and PEI-Env plasmid DNA groups showed poor immune responses, even lower than that of the naked Env plasmid DNA group (Figure 4A). There were no immune spots when mice were injected with PDDAC-Au NR (data not shown), which meant that the cellular immune response was antigen-specific but not carrier-specific. Meanwhile, the titer of specific antibody for Env DNA antigen was enhanced significantly in the PDDAC-Au NR-Env (nearly 8 times, titer-1:25600) and PEI-Au NR-Env plasmid DNA groups (nearly 4 times, titer-1:12800) compared to the naked Env plasmid DNA group (titer-1:3200) (Figure 4D).

To protect organisms from the attacks of external and internal pathogens, humoral and cellular immunity are the primary and pivotal defensive barriers, respectively. The most important role of a vaccine is to make the immune response of the organism occur rapidly and strongly upon a second exposure to the same pathogen. T cell proliferation capability can indicate the immune state of the organism indirectly. Results of the T cell proliferation assay demonstrated that the PDDAC-Au NR-Env and PEI-Au NR-Env groups had perfect T cell proliferation capacity compared to the naked Env plasmid DNA group (Figure 4B,C). In other words, after immunization with PDDAC-Au NR-Env or PEI-Au NR-Env, the CD3⁺CD8⁺ T cells could proliferate rapidly and eliminate the infected cells when mice encountered the pathogen again. Meanwhile, the CD3⁺CD4⁺ T helper cells could also proliferate quickly and regulate the CTL and B cell functions. The histograms for T cell proliferation are available in Supporting Information (Figure S8, Supporting Information).

To evaluate the bias of the immune response, we used the Th1/Th2 ratio. Th1 cells drive cellular immunity to fight viruses and other intracellular pathogens, while Th2 cells drive humoral immunity to eliminate extracellular pathogens. In mice, Th1 and Th2 cells induce the production of IgG2a and IgG1, respectively. Results for the Th1/Th2 ratio demonstrated that the value of [IgG1]/[IgG2a] in the PDDAC-Au NR-Env group increased significantly compared to the naked Env
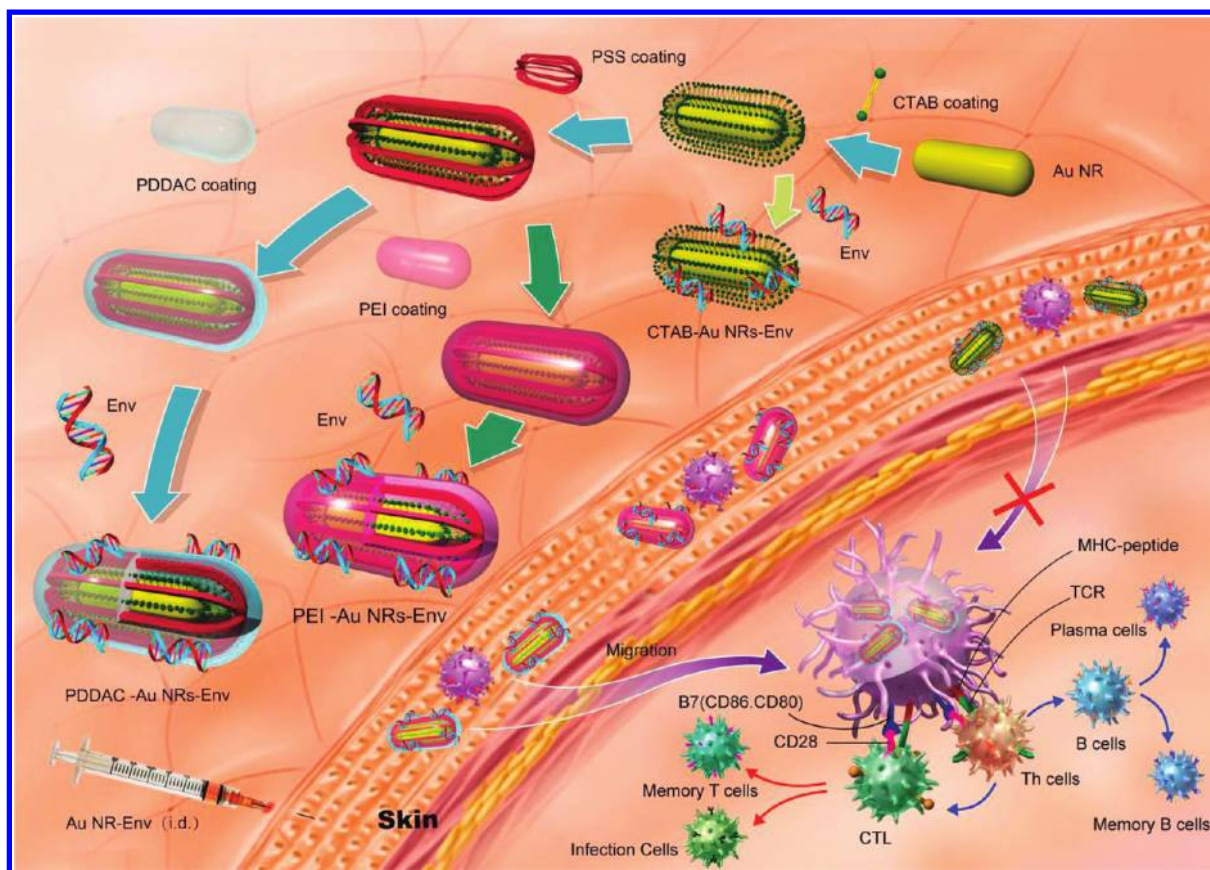
Figure 5. The possible mechanism of Au NRs as vaccine adjuvants. Au NRs with different surface coatings mixed with Env and the Au NR-Env complex formed. After intradermal injection of PDDAC- and PEI-Au NR-Env complexes, APCs, especially DCs located in the skin (Langerhans cells), can phagocytose the Au NR-Env complex, process the antigens during the migration to secondary lymph nodes, and become mature while presenting the MHC-peptide complex to native T cells upon arrival at lymph nodes. At this moment, B7 molecules (CD80, CD86) are highly expressed on the surface of DCs. CTL will proliferate rapidly and eliminate intracellular pathogens under the regulation of activated Th1 cells. Meanwhile, there is also the possibility that Th1 polarization occurs first and then transforms to Th2 polarization. The activated Th2 cells will induce B cell transformation to plasma cells, which secrete immunoglobulin (IgG). Subsequently, some of the T and B cells will become memory cells to protect the organism upon a second encounter with the same pathogen. However, CTAB-Au NR inhibited the DC maturation. In turn, it could not improve the immunogenicity of HIV-1 DNA vaccine.

plasmid DNA group (Figure 4E), which indicates a Th2-biased immune response.

Possible Mechanism for Au NRs as Adjuvants - Promoting DC Maturation. Our in vivo results demonstrated that Au NRs with different surface coatings showed different immunological effects. What is the mechanism for Au NRs as vaccine adjuvants and how do Au NR-Env complexes trigger the immune response? Antigen-presenting cells (APCs), especially dendritic cells, play pivotal roles in linking innate and adaptive immunity; they govern the initiation of both humoral and cellular immunity. As the first signal, the interaction between MHC-peptide complexes expressed on APCs and T cells plays a critical role in cellular immunity. However, a second signal or costimulatory molecules such as the B7 molecules (CD86 and CD80) or others delivered by the same APC are required to trigger clonal expansion and differentiation of a naive T cell. Since intradermal injection was chosen as the immunization route and the skin is rich in APCs, Au NR or the Au NR-Env complex must interact with DCs. In order to test this interaction, we cultured bone marrow-derived dendritic cells (BM-DCs) from BALB/c mice according to the method of Manfred B. Lutz.[45] We found that the percentage of mature DCs ($CD11c^+MHCII^+CD86^+CD80^+$ DCs) increased significantly when DCs were treated with

PDDAC-Au NR, the PDDAC-Au NR-Env complex, PEI-Au NR or the PEI-Au NR-Env complex, but not CTAB-Au NR or CTAB-Au NR-Env compared to the untreated DCs (Figure 4F). That is to say, all the other Au NRs and Au NR-Env complexes can significantly enhance DC maturation except CTAB-Au NR and the CTAB-Au NRs-Env complex. CTAB-Au NR also exhibited toxicity when HEK293 cells were treated with it for 3 h (Figure 3D), which would explain why only CTAB-Au NR cannot promote immune responses.

From the above results, a conclusion could be drawn that the surface chemistry greatly determined the adjuvant activities of the gold nanorod. Mechanism underlying Au NRs as vaccine adjuvants can be shown as Figure 5. First, Au NRs with different surface coatings mixed with Env and the Au NR-Env complex formed. After intradermal injection with PDDAC- and PEI-Au NR-Env complexes, APCs, especially DCs located in the skin (Langerhans cells), phagocytose the Au NR-Env complex, process the antigens during the migration to secondary lymph nodes, and become mature while presenting the MHC-peptide complex to native T cells upon arrival at lymph nodes. At this moment, B7 molecules (CD80, CD86) are highly expressed on the surface of DCs. CTL will proliferate rapidly and eliminate intracellular pathogens under the regulation of activated Th1 cells. Meanwhile, there is also the

possibility that Th1 polarization occurs first and then transforms to Th2 polarization. The activated Th2 cells will induce B cell transformation to plasma cells, which secrete immunoglobulin (IgG). Subsequently, some of the T and B cells will become memory cells to protect the organism upon a second encounter with the same pathogen. However, CTAB-Au NR inhibited the DC maturation. In turn, it could not improve the immunogenicity of HIV-1 DNA vaccine.

Because of their properties like enhancing the cellular uptake of functional molecules (e.g., DNA), protecting drugs and genes from degradation, and easy preparation and modification, nanomaterials show great potential in improving the immunogenicity of DNA vaccines. Although some different kinds of nanomaterials have been exploited as vaccine carriers/adjuvants, there is lack of intended design to improve the immune response. Hence, systematic investigation is urgently needed. In this study, results demonstrated that surface chemistry had great influences on transfection capabilities of gold nanorods and finally determined their adjuvant activities. These results will provide useful information and shed light for the rational design of low-toxic nanomaterials as safe vaccine adjuvant/delivery systems.

Although previous evidence suggested that PEI could enhance the immune response,[46] its poor reproducibility and the acute cytotoxicity restricted its clinical development.[47] The toxicity could be decreased when PEI was conjugated to other polymers but maintaining a good transfection capability.[48] In the present study, PEI-Au NRs surpass PEI as tranfection reagents and vaccine adjuvant/delivery systems. The promoting effects would be from the combining roles of PEI surface and Au nanorods. The present data suggest that PDDAC-Au NR triggered Th2-biased immune response. But it should be noted that the hypothesis of Th1/Th2 balance sometimes has inconsistencies since the nonhelper regulatory T cells, or the antigen-presenting cells (APC), likely influence immunity in a manner comparable to Th1 and Th2 cells. Many diseases previously classified as Th1 or Th2 dominant fail to meet the set criteria. Experimentally, Th1 polarization is readily transformed to Th2 dominance through depletion of intracellular glutathione and vice versa.[49]

While the properties of the vaccine carrier or adjuvant are vital, the immunization route also plays an important role in improving the efficiency of a DNA vaccine. We have immunized mice through subcutaneous injection (s.c.), intramuscular injection (i.m.), intraperitoneal injection (i.p.) and intranasal injection (i.n.). However, there were no promising results for these four immunization methods. For instance, there were few immunity spots in the experimental groups and no significant differences compared to the naked Env plasmid DNA group for these injection methods. In fact, the muscle is not an efficient site for antigen presentation due to the lack of suitable quantities of dendritic cells, macrophages, or lymphocytes.[50] Therefore, intramuscular injection may be not the optimal administration method for these vaccines. Compared to muscle, the skin is one of the largest immune organs and is rich in potent antigen-presenting cells such as immature Langerhans cells in the epidermis and mature DCs in the dermis;[51] it also contains other cells that are important in helping to elicit immune responses.[52] However, subcutaneous injection cannot take full advantage of the immune system of the skin, which may explain why gold nanorods exhibit adjuvant activity only through the intradermal route.

Activation of the immune system by a vaccine requires (i) the delivery of a sufficient amount of antigen to antigen presenting cells (APCs), (ii) the controlled presentation of antigen to target immune cells ($CD4^+$ and/or $CD8^+$ T cells), (iii) the proliferation of effector cells such as cytotoxic T lymphocytes (CTLs) and plasma B cells, and (iv) the maintenance of an activated immune system for the desired period of time.[53] Edelman[54] classified adjuvants into three groups including (i) active immunostimulants, being substances that increase the immune response to the antigen; (ii) carriers; and (iii) vehicle adjuvants, which serve as a matrix for antigens as well as stimulating the immunity. In this study, Au NRs with proper coatings can play two roles in promoting DNA vaccine immunogenicity. First, Au NRs modified with proper surface coating can act as an effective carrier to enhance the uptake of DNA (pEGFP, Env plasmid DNA) into cells, including somatic cells and APCs. Second, they promote DC maturation to initiate and amplify the immune responses. Usually, most vaccine adjuvants do not act directly on T cells, but exert their functions indirectly via effects on APCs. Among APCs, DCs, recently a new focus in vaccine development, are the most effective at inducing activation and proliferation of native T cells in vitro and in vivo. However, our present data show that HIV Env plasmid DNA delivered by PDDAC- and PEI-Au NRs can activate T cell proliferation greatly and facilitate DC mature directly, which may provide promising candidate adjutants for clinical application. Thus, the detailed mechanisms of gold nanorods as adjuvant need further investigation in future.

Moreover, the vaccine adjuvant is usually designed with versatile functions, including enhancing the immunogenicity of antigens, promoting immune responses especially for low responder individuals, decreasing the usage of antigens, and reducing immunization times. Additionally, gold nanorods show aspect ratio-dependent SPR features and make a strong SPR response in near-infrared spectral region, which would be also useful for further flexible applications, like light-mediated release of DNA vaccine and imaging.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The methods and experimental procedures are provided. The images of cell transfection at 24 h post transfection of Au NRs, the characterizations of Au NR and Au NR-DNA complexes with a UV−vis-NIR spectrometer and Zetasizer, the colocalizations of the Au NR-DNA complex and lysosomes, the dynamic localization of Au NR-DNA complexes and mitochondria, TEM images for Au NRs-DNA complexes at different time points post-treatment, the CD spectra of plasmid DNA treated with gold nanorods, the paradigm for the possible mechanism of three kinds of Au NRs, and the histogram of T cell proliferation for all of the groups are also provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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