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Research paper

Controlled release of PEI/DNA complexes from PLGA microspheres as a potent delivery system to enhance immune response to HIV vaccine DNA prime/MVA boost regime

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Abstract

A novel approach involving the preparation of biodegradable PLGA microspheres containing entrapping complexes of DNA and polyethylenimine was developed to improve the delivery of DNA into antigen-presenting cells after intramuscular injection. Compared to the traditional biodegradable microspheres which release naked DNA, these microspheres released intact and penetrative PEI/DNA complexes over a period of 2 weeks *in vitro*. In addition, the DNA was not degraded during encapsulation in the PLGA microspheres, owing to the protection of polyethylenimine. After i.m. immunization, the microspheres induced significantly enhanced serum antibody responses 2–3 orders of magnitude greater than naked DNA. Additionally, in contrast to naked DNA, the microspheres induced potent CTL responses at a low dose.

Keywords: PLGA microspheres; Solvent emulsification-diffusion; DNA vaccine; PEI/DNA complexes; Controlled release; Immunogenicity

1. Introduction

Cytotoxic T lymphocytes (CTL) are associated with the control of viremia in human immunodeficiency virus type 1 (HIV-1)-infected patients [1,2] and simian immunodeficiency virus (SIV)-infected monkeys [3–5]. However, currently available therapeutic approaches do not induce HIV-1-specific immunity. On the contrary, $CD4^+$- and $CD8^+$-mediated T-cell responses decline in time in patients treated with high active antiretroviral therapies [6]. The absence of HIV-1-specific cellular immunity contributes to treatment failures and to viral rebound after interruption of therapy. Encouraging results have recently indicated that HIV-1-specific T-cell responses could be enhanced with a heterologous DNA prime/MVA boost immunization regime [7,8]. These results provide a rationale to develop the HIV-specific immunity, however, 1–2 mg of DNA was utilized in these studies. Although the use of DNA vaccines at milligram doses is feasible, it would impose serious limitations on the number of constructs that could be included in a vaccine [9]. In addition, the use of very high doses of DNA is less favorable from a

Abbreviations: PLGA, poly(D,L-lactide-co-glycolide); APCs, antigen-presenting cells; i.m., intramuscular; DCs, dendritic cells; CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; MΦs, macrophages; PEI, polyethylenimine; DLS, dynamic light scattering; SEM, Scanning electron microscopy; TA, anterior tibialis; LDH, lactate dehydrogenase.

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process economics standpoint. There is, therefore, a clear need to induce effective immunity with lower and fewer doses of DNA, as well as to increase the magnitude of immune responses obtained.

The technology that formulated DNA into PLGA microspheres was developed as a means to better target DNA to antigen-presenting cells (APCs). PLGA microspheres are an attractive approach for vaccine delivery, since the polymer is biodegradable and biocompatible and has already been used to develop several DNA vaccine delivery systems [10,11]. During microencapsulation, however, the DNA is exposed to a range of conditions that have the potential to cause denaturation and degradation, including high shear, an organic/aqueous interface, localized high temperature, and freeze drying. Not surprisingly, recent observations have shown that DNA is damaged during microencapsulation, leading to a significant reduction in supercoiled DNA [12,13].

To overcome the problems of DNA degradation during microencapsulation, we have developed a novel approach to incorporate PEI/DNA complexes into PLGA microspheres. PEI spontaneously condenses DNA and transforms the filiform DNA molecules into a compact particle, improving both chemical and physical properties. Additionally, PEI buffers the acidity of endosomes and confers the PLGA microsphere system with a strong proton buffer capacity over a broad pH range. After preparation and characterization, the PLGA microspheres were administrated to experimental animals and the immune responses induced were compared with those of naked DNA.

2. Materials and methods

2.1. Materials

Poly(DL-lactide-co-glycolide) (PLGA, RG505), 25 kDa PEI (branched), Poly(vinyl alcohol) (PVA) were purchased from Sigma–Aldrich. The HIV-1 D-GPEi plasmid (a coden-optimised DNA plasmid containing the Gag, Pol and Env gene in eukaryotic vector VR 1012, subtype recombinant B/C, 13,113 bp), pCMV Luc plasmid (encoding luciferase driven by the cytomegalovirus (CMV) promoter, 6.4 kb) were constructed by our Lab. The plasmids were purified by using a Qiagen Plasmid Giga kit, and the final product was endotoxin free (<2.5 U/mg).

2.2. Methods

2.2.1. PLGA microspheres synthesis and characterization

PLGA microspheres were prepared by using a modified solvent evaporation process (Scheme 1). Briefly, the microspheres were prepared by emulsifying 10 ml of a 6% (wt/vol) PLGA solution in methylene chloride with 2 ml of PEI/DNA complexes at high speed using a homogenizer. The primary emulsion then was added to 50 ml of distilled water containing PVA (1%, wt/vol). This resulted in the formation of a water/oil/water emulsion that was stirred for 12 h at room temperature, allowing the methylene chloride to evaporate. The resulting microspheres were washed twice in distilled water by centrifugation at 10,000*g* and freeze-dried. Fluorescent-labeled microspheres were prepared by adding 6-coumarin (5 mg/g polymer, Acros Organics) to the organic phase.

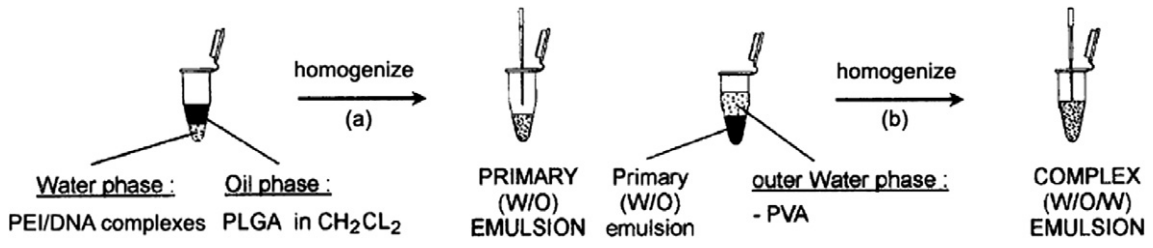
The size distribution of the microspheres was determined by dynamic light scattering (DLS) with a Zetasizer 3000 HS (Malvern Instruments, UK) and the value was calculated by volume measurement. The loading level of DNA in the microspheres was determined by assaying the supernatant after centrifugation and measuring DNA by absorbance at 260 nm. The encapsulation efficiency of microspheres was calculated. The Zeta potential of the microspheres was measured on the same apparatus as DLS. Selected batches of microspheres were evaluated by scanning electron microscopy (SEM) for size and surface uniformity.

2.2.2. In vitro release study

In vitro release studies of DNA from microspheres were carried out by suspending 10 mg of microspheres in 1 ml of PBS at pH 7.4 (120 mM NaCl, 2.7 mM KCl, 10 mM PBS) at 37 °C under stirring. At predetermined time intervals, the suspension was centrifuged and replaced with the same volume of fresh medium. The DNA concentration in the supernatant was determined by UV absorbance at 260 nm. Each experiment was performed in triplicate.

2.2.3. Plasmid DNA stability evaluation

CD (Circular Dichroism) spectra: The conformation of free DNA, PEI/DNA and PEI/DNA released from PLGA microspheres was derived by the simple inspection of their CD spectra. The CD spectra were obtained at 25 °C.



Scheme 1. Schematic representation of microencapsulation procedures for PEI/DNA complexes containing PLGA microspheres.

Transfection *in vitro*: for transfection *in vitro*, COS-7 cells were cultured in DMEM containing Gln supplemented with 10% heated-inactivated FCS and antibiotics. Cells were grown at 37 °C in humidified air containing 5% CO_2 and passaged every 2–3 days. The cells (1×10^5 per well) were plated on 12-well tissue-culture plates 24 h before transfection. Immediately before the initiation of transfection experiments, the medium was removed from each well, and the cells were washed once with DMEM without serum and antibiotics and treated with PEI/DNA complexes or PEI/DNA released from PLGA microspheres. Forty-eight hours later the cells were harvested and luciferase gene expression was determined by using a commercial kit (Promega). Each transfection experiment was done in triplicate and is expressed as mean light units per mg of cell protein ± SD.

2.2.4. In vitro targeted delivery

RAW264.7 cells (murine monocyte-macrophage cells) were used 2–3 days after seeding for targeted delivery studies. Fluorescent-labeled microspheres were suspended in cell culture medium and added to the cells at a concentration of 1×10^5 particles/well in a 24-well plate. HeLa cells were used as controls. The cells were incubated for 2 h at 37 °C. They were then washed with DMEM and fixed with 3% paraformaldehyde in PBS prior to microscopic analysis.

2.2.5. Gene expression: in vivo

Two groups of female BALB/c mice ($n = 6$) were injected with either 50 μg of pCMVLuc DNA or the PLGA microspheres containing 50 μg of pCMVLuc DNA. Both groups of mice were injected i.m. in the anterior tibialis (TA) muscle on either leg. The TA muscle from each mouse in two groups was harvested either at day 1, 7, or 14, and stored in a −80 °C freezer. The muscles were ground with a mortar and pestle on dry ice, then collected in Eppendorf tubes with 0.5 ml of 1 × reporter lysis buffer. After freeze/thawing three times, the samples were spun at 14,000 rpm for 10 min, and the supernatants collected. Within each group, the supernatants at each time point were pooled, and 20 μl of the samples was assayed by using the luciferase assay, and normalized to the total volume.

2.2.6. Mice

BALB/c mice were bred and maintained in the animal facilities of the vaccine research center of Jilin University, Changchun. Only female mice 6 to 8 weeks old were chosen for the first vaccination. Permission to do animal work was obtained from the Laboratory Animal Ethics Committee of Jilin University, China.

2.2.7. Humoral immune response

Female BALB/c mice in groups of 8 were immunized with either the PLGA microspheres, naked DNA, or control PBS at different doses (0.1, 1, 10 or 100 μg). The immunization protocol was performed at weeks 0, 4 and boosted with MVA at week 8. Sera samples were collected from all mice by tail bleeding at time points and HIV-1-specific serum IgG titers were confirmed by Western blotting.

2.2.8. Cellular immune response

Spleens from immunized mice were harvested 5 days after the third immunization and used in pools of five. Spleen cells were cultured with RMPI 1640 phenol red-free medium containing 10% FCS. Approximately 1×10^6 P815/BALB target cells were sensitized with p7g peptide (amino acids 194–213) at a concentration of 1 mM for 2 h at 37 °C. Cytotoxicity was measured by a standard LDH (lactate dehydrogenase)-release assay with a CTL kit (Promega).

3. Results and discussion

3.1. PLGA microspheres evaluation

Scanning electron microscopy showed that freshly prepared PLGA microspheres are fairly spherical (Fig. 1a).

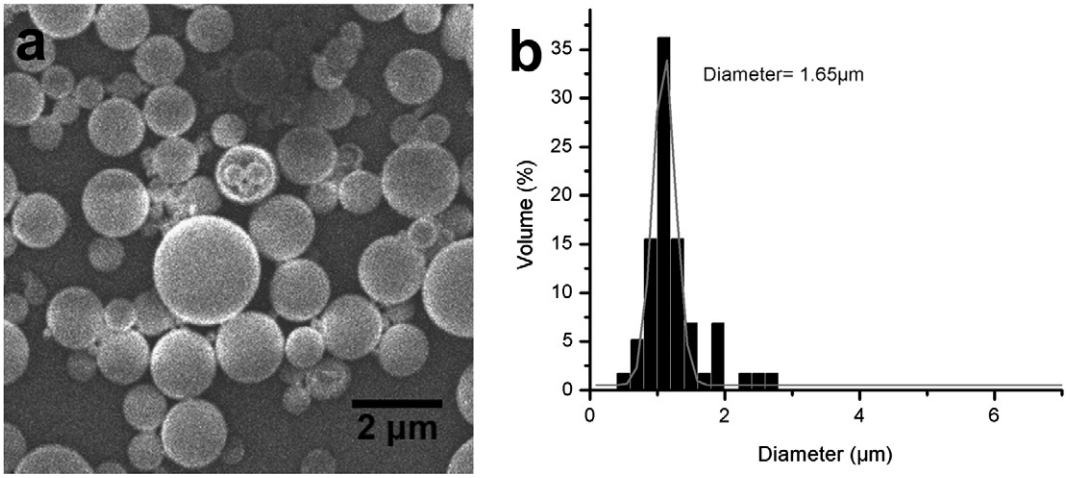


Fig. 1. Appearance and size distribution of PLGA microspheres. (a) Scanning electron micrograph of PLGA microspheres. Scale bar represents 2 μm. (b) Size distribution of freshly prepared microspheres. Size was measured using photon correlation spectroscopy (dynamic light scattering) and data were plotted as volume distribution.

The PLGA microspheres had a mean size of approximately 1.65 μm and showed a unimodal particle size distribution (Fig. 1b). The surfactant or stabilizer most commonly used during PLGA microspheres preparation, polyvinyl alcohol, imparted a negative surface charge on the microspheres, because of physical entrapment within the surface layers of the polymer.

3.2. In vitro release

The release profiles of PEI/DNA from PLGA microspheres can be presented as two phases (Fig. 2): fast release (48.9% of the DNA encapsulated at the first 6–7 days) and sequential slow release. The fast release may be due to some PEI/DNA complexes sitting on or imbedded in the PLGA microspheres surface or in an imperfection of the composite surface. The mechanism of slow release is relatively complicated. It may involve the following aspects: (i) water permeation through the PLGA matrix and diffusion of DNA through the complex, water-filled path in the PLGA microsphere matrix; (ii) PLGA degradation.

3.3. DNA stability

Since plasmid DNA is unstable during PLGA microspheres, we hypothesized that PEI could spontaneously condense DNA and transform the filiform DNA molecules into a compact particle to improve both chemical and physical properties. To test this hypothesis, the conformation of plasmid DNA after homogenization was checked by CD spectrum analysis. The CD spectrum of DNA or PEI/DNA is typical of a duplex in the "B" conformation. PEI/DNA complexes released from PLGA microspheres resulted in CD spectra with similar conformations, indicating that the DNA retained its helical structure (Fig. 3a).

Preservation of bioactivity of DNA is even more important than preservation of conformation. *In vitro* gene expression studies were performed on pLuc released from PLGA microspheres, to confirm that the DNA released was intact and able to be expressed in cells. The pLuc released from the microspheres *in vitro* produced expression levels in COS-7 cells analogous to the untreated plasmid control (Fig. 3b), providing that the DNA released from the microspheres was bioactive and could be expressed in normal cells.

3.4. In vitro targeted delivery studies

The PLGA microspheres fluorescent-labeled with coumarin were added to either the mouse monocyte-macrophage cell line (RAW264.7) or human epithelial cells (HeLa) to determine whether the microspheres could be internalized. Efficient internalization of PLGA microspheres was found in RAW264.7 cells (Fig. 4a and b) and no sphere uptake was obtained in HeLa cells (Fig. 4c and d). Spheres in range of 0.5–3 μm are too large to enter the "ordinary" cells via endocytosis, and therefore "target" phagocytic cells such as macrophages, by size exclusion. Because macrophages are antigen-presenting cells in the

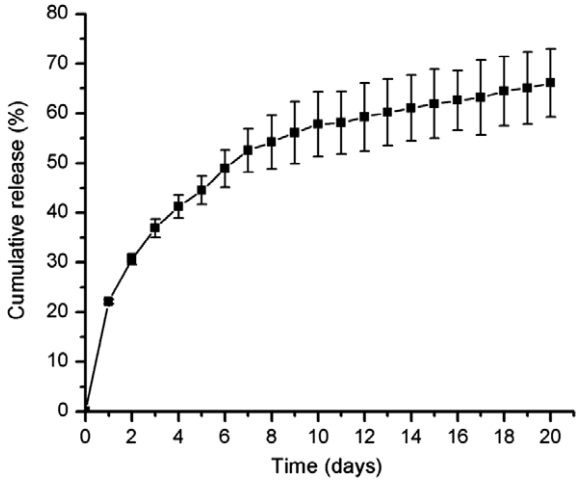


Fig. 2. *In vitro* release from PLGA microspheres in PBS pH 7.4 at 37 °C. The microspheres (10 mg) were suspended in 1 ml of PBS at pH 7.4 (120 mM NaCl, 2.7 mM KCl, 10 mM PBS) at 37 °C under stirring. At predetermined time intervals, the suspension was centrifuged and replaced with the same volume of fresh medium. The DNA concentration in the supernatant was determined by UV absorbance at 260 nm. All data were expressed as mean ± SD ($n = 3$).

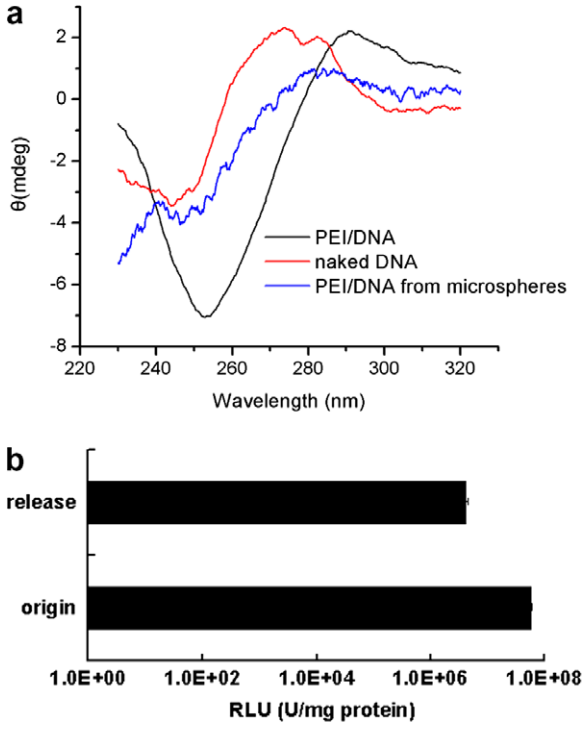


Fig. 3. (a) Circular dichroism spectrum of naked DNA, PEI/DNA and PEI/DNA released from PLGA microspheres. (b) *In vitro* gene expression of DNA released from PLGA microspheres. Mean ± SD of triplicate reading is represented.

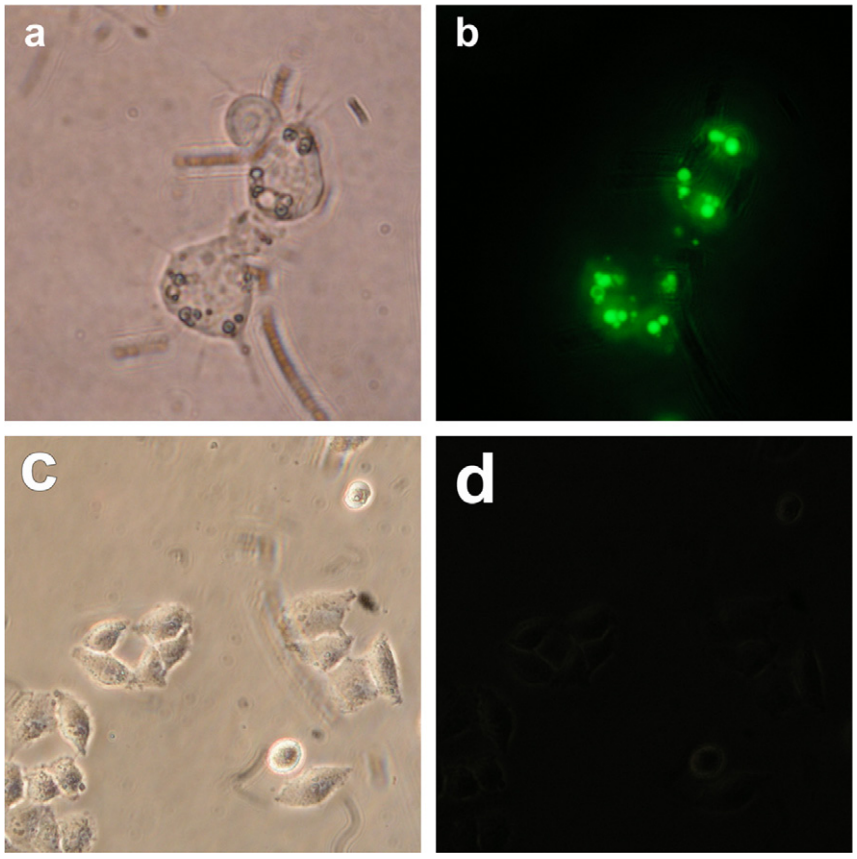


Fig. 4. Phagocytosis of PLGA microspheres by MOs. (a and b) Uptake of fluorescent-labeled PLGA microspheres after 4 h of incubation at 37 °C. Cells were examined simultaneously by phase contrast (left panel) and fluorescence (right panel). (c and d) Control experiments were performed with HeLa cells showing no particle uptake.

immune system, microencapsulated plasmid DNA is particularly useful for the rapidly developing field of DNA vaccines.

3.5. Gene expression after intramuscular injection

The PLGA microspheres induced expression of luciferase *in vivo* after injection into TA muscle in BALB/c mice. The level of *in vivo* expression of luciferase was higher for PLGA microspheres than for naked DNA at the days 7 and 14 (Fig. 5). Additionally, after dosing the muscle, the overall expression levels achieved using the PEI/DNA based formulation were lower than those achieved with PLGA microspheres. It is interesting that *in vitro*, the PEI performed much better than PLGA microspheres (data not shown); however, *in vivo*, the PLGA microspheres performed better than the PEI/DNA formulation (Fig. 5). This inconsistency between *in vitro* and *in vivo* performance has been highlighted previously [14]. We hypothesize one reason why PEI formulations work well *in vitro* but not necessarily *in vivo*. Complement system activation may explain the low gene expression level *in vivo*. PEI/DNA complexes may undergo rapid inactivation due to undesired interactions with components of the *in vivo* milieu. Generally, polycations are less immunogenic; however, the activation of innate defense mechanisms is still a major problem. Complement system activation by PEI has been demonstrated, with highly positive complexes being the most active stimulators [15]. In our study, the negatively charged microspheres might be shielding the positive PEI/DNA particles, thereby avoiding activation of the complement cascade (Table 1).

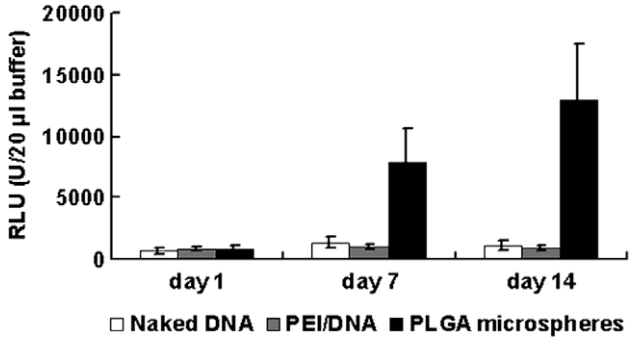


Fig. 5. *In vivo* gene expression of luciferase was performed in mice at days 1, 7 and 14 with either naked DNA, PEI/DNA complexes or PLGA microspheres. Mean average values ± standard deviation of triplicates are given in relative light units (RLU), correlated to the total volume content.

Table 1
PLGA microspheres encapsuled PEI/DNA within it

Formulation	Mean size[a] (nm)	Encapsulation efficiency[b] (%)	Zeta potential (mV)
PLGA	1650 ± 1086	(41.7 ± 7.8)%	−29.44 ± 7.78
PEI/DNA (N/P = 6)	86.6 ± 30.2		36.64 ± 1.77

[a] Obtained from DLS.
[b] The encapsulation efficiency was determined by assaying the supernatant after centrifugation and measuring DNA by absorbance at 260 nm. All data were expressed as mean ± SD ($n = 3$).

3.6. Evaluation of immune responses

To assess the relative ability of a promising DNA vaccine technology to prime antibody and T-cell responses, BALB/c mice were immunized with HIV-1 Gag DNA with and without formulation into PLGA microspheres.

3.6.1. Enhanced HIV-1-specific humoral responses

The PLGA microspheres induced significantly enhanced antibody responses over naked DNA at all time points and at three doses evaluated (Fig. 6a). In an extensive dose–response titration assessed by Western blotting, naked DNA primed HIV-1-gag-specific antibody response after priming with DNA as high as 100 μg and boosted with MVA (compared with the negative controls PBS-treated mice and blank microspheres treated mice). In contrast, PLGA/PEI/DNA microspheres were effective at 0.1 μg. This indicates a ~1000-fold increase in DNA vaccine potency, as judged by the lowest dose of DNA required to prime a measurable response. The PLGA microspheres may increase the humoral response of naked DNA by extending the kinetics of its release time.

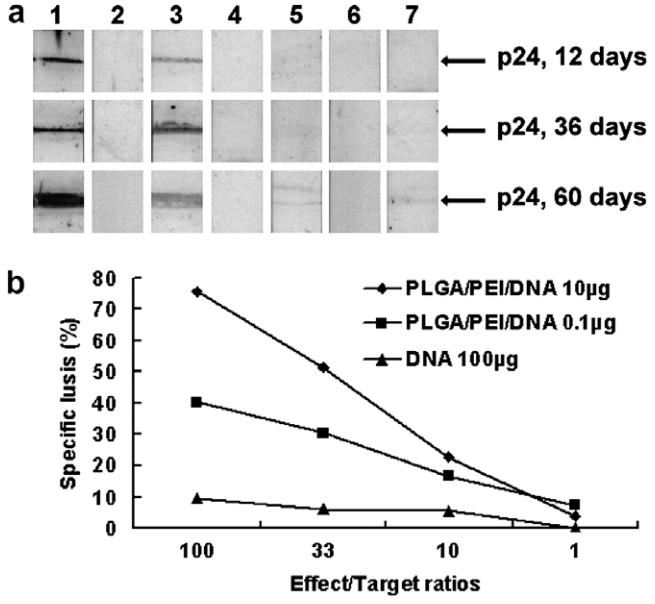


Fig. 6. Mice were primed at weeks of 0 and 4 and boosted at week 8 with MVA. (a) Western blotting analysis of mice serum antibody responses to HIV-1 p24 antigens. Mice sera collected at the indicated (at days 12, 36 and 60) after injection were tested at 1:40 dilution (as pools of five) 1, 3, 5. PLGA microspheres with different DNA doses (10, 1, 0.1 μg); 2, 4, 6, 7, naked DNA at different doses (10, 1, 0.1, 100 μg). (b) Analysis of cytolytic activities of splenic mononuclear cells harvested from immunized mice. P815 mastocytoma cells pulsed with epitope peptides were used as targets. Percent-specific lysis was determined by a 4-h LDH-release assay.

3.6.2. Facilitated HIV-1-specific CTL responses

To quantify T-cell responses, mice were boosted with a recombinant vaccinia virus expressing Gag. Five days later, HIV-1-specific $CD8^+$ T cells were measured *in vitro* by assessing the cell death of P815/BALB target cell, which were sensitized with an H-2^d-restricted CTL epitope. Naked DNA primed mild Gag-specific $CD8^+$ T cells response at the dose of DNA as high as 100 μg. In contrast, PLGA/PEI/DNA microspheres were more effective at 100 ng, indicating a >1000-fold increase in DNA vaccine potency, as judged by reduction of DNA vaccine dose (Fig. 6b).

Our results demonstrate that the multiple PLGA microspheres are potent delivery systems for DNA vaccines, and induce significantly enhanced antibody and CTL responses to HIV vaccine DNA prime/MVA boost regime. Furthermore, the levels of immune enhancement achieved in the current studies appeared much greater than previously reported with alternative PLGA formulations. In previous study involving DNA entrapped in PLGA microspheres, no enhancement of antibody responses was reported [10]. In addition, in that study, the PLGA microspheres were administered by a different route than the naked DNA, preventing a direct comparison of CTL induction [10]. In our studies, the PLGA microspheres were immunized by the i.m. route, which is optimal for immunization with naked DNA.

The mechanism of the adjuvant effect achieved with PLGA microspheres is not currently known, but we believed that efficient delivery of entrapped DNA to APCs is an important contributing factor. The theory is supported by the observation that PLGA microspheres are too large to enter ordinary cells via endocytosis, and therefore target phagocytic cells such as macrophages by size exclusion. Previous studies have shown that bone marrow-derived APCs are essential to present antigen encoded by DNA vaccines to naive CTL precursors, possibly by transfer of antigen from muscle cells to APCs, a process referred to as cross priming. Although the mechanism of DNA vaccination has not been fully elucidated, recent studies have suggested an important role for directly transfected APCs after immunization [16]. Furthermore, the presence of the cationic polymer PEI may make an additional contribution to the mechanism by contributing to disruption of endosomes and the release of DNA into the cytoplasm. However, this hypothesis remains to be proven and further studies are necessary.

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