Abstract

DNA vaccines are simple to produce and can generate strong cellular and humoral immune response, making them attractive vaccine candidates. However, a major shortcoming of DNA vaccines is their poor immunogenicity when administered intramuscularly. Transcutaneous immunization (TCI) via microneedles is a promising alternative delivery route to enhance the vaccination efficacy. A novel dissolving microneedle array (DMA)-based TCI system loaded with cationic liposomes encapsulated with hepatitis B DNA vaccine and adjuvant CpG ODN was developed and characterized. The pGFP expression in mouse skin using DMA was imaged over time. In vivo immunity tests in mice were performed to observe the capability of DMA to induce immune response after delivery of DNA. The results showed that pGFP could be delivered into skin by DMA and expressed in skin. Further, the amount of expressed GFP was likely to peak at day 4. The immunity tests showed that the DMA-based DNA vaccination could induce effective immune response. CpG ODN significantly improved the immune response. The cationic liposomes could further improve the immunogenicity of DNA vaccine. In conclusion, the novel DMA-based TCI system can effectively deliver hepatitis B DNA vaccine into skin, and induce effective immune response.

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Keywords: Dissolving microneedle arrays, DNA vaccine, Adjuvants, CpG ODN, Cationic liposomes
1. **Background**

Despite the potential attractive features of DNA vaccines, their use in clinical settings remains limited because of their poor immunogenicity, especially in non-human primates and humans. Transcutaneous immunization (TCI) is receiving increased attention as an alternative route of immunization. In the TCI procedure, the skin is the target for vaccination. The skin has important immune functions, because the epidermis and dermis are highly populated by dendritic cells (DCs) and Langerhans cells (LCs), which can capture and present the antigens (Ag) to the lymph and initiate the immune response. Therefore, it is possible that DNA vaccine delivery would be substantially enhanced by target DNA to the highly immunoresponsive layers of the skin.

However, the outer stratum corneum layer of skin represents a significant barrier to the delivery of genes and other high molecular weight agents, so improved delivery strategies are required to overcome this skin barrier property. Recently, development of microneedles has enabled painless cutaneous delivery of various vaccines to skin layers with resident professional APCs. In this paper, we developed a novel DMA system to improve the immunogenicity of HBV DNA vaccine. Likewise, cationic liposomes encapsulated with HBV DNA vaccines and adjuvant CpG ODN was loaded into the DMA for a further improvement of immunogenicity. The gene expression in the skin by DMA was investigated. And the immune effect was evaluated and compared with conventional intramuscular injection in vivo.

2. **Results and Discussion**

2.1 **Preparation and characterization of liposomes**

The plasmid DNA vaccine VR-E2E that containing a plasmid vector VR2012 encoding the middle (pre-S2 plus S) envelope proteins of HBV was prepared in our lab. The reporter plasmids pGFP was amplified and purified from previously transformed stocks of Escherichia coli DH5α using antibiotic selective conditions and a QIAGEN® Mega Kit (QIAGEN Ltd, Crawley, UK). The cationic liposomes containing the VR-E2E (Lip+VR-E2E) were prepared as modified by previously described. The cationic liposomes containing VR-E2E plus CpG ODN (Lip+VR-E2E+CpG), or pGFP (Lip+pGFP) were prepared following the same method. The final concentrations of VR-E2E, pGFP, CpG ODN in the liposomes were 0.5mg/ml, 0.5mg/ml, 1mg/ml, respectively. The morphology of Lip+VR-E2E+CpG in dissolving microneedles was observed by Scanning Electronic Microscopy (SEM). The zeta potential and particle size of all liposomes were determined by Zetasizer 3000HS (Malvern, UK).

The morphology of empty liposomes, and Lip+VR-E2E+CpG ODN in solution or recovered from the DMA, was shown in Fig. 1. These appeared as spherical structure, confirming the vesicular characteristics. The vesicle size and the polydispersity of size distribution of the liposome formulations measured by DLS were shown in Table 1. The size of liposomes were increased after loaded with VR-E2E and CpG ODN (P<0.05), no matter in the fresh solution or in the DMA. And the size of liposomes was also increased after loading in the DMA than that in the fresh solution (P<0.05). Meanwhile, the polydispersity (PDI) was significantly increased after loaded with VR-E2E and CpG ODN. It may be due to the lower zeta potential, which leading to a weaker repulsive force between liposomes. And the PDI was significantly decreased after loaded in DMA than in fresh solution. It may be attributed to that PVP in the DMA is a good dispersant.
Fig. 1 Visualization of cationic liposomes by scanning electron microscope (50,000×). A: Empty Liposome; B: Lip+VR-E2E+CpG OND in solution; C: Lip+VR-E2E+CpG OND loaded in DMA.

As it shown in Table 1, the ability of the cationic liposomes to entrap VR-E2E and CpG ODN in solution and in DMA was investigated. It was shown that the entrapment of VR-E2E and CpG ODN by cationic liposomes in solution was 88.3 ± 2.6% and 86.3 ± 3.4%, respectively. Simultaneously, the entrapment of VR-E2E and CpG ODN by cationic liposomes in DMA was declined to 58.3 ± 3.1% and 62.3 ± 4.2%, respectively. It suggested that the cationic liposomes had similar ability to entrap VR-E2E and CpG ODN, no matter in solution or in DMA.

Table 1. Characterizations of different Liposome formulations.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Polydispersity</th>
<th>Zeta Potential (mV)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Lip*</td>
<td>0.44 ± 0.03</td>
<td>42.4 ± 6.9</td>
<td>140.0 ± 1.1</td>
</tr>
<tr>
<td>Lip+VR-E2E+CpG OND*</td>
<td>0.82 ± 0.03</td>
<td>38.7 ± 3.5</td>
<td>198.5 ± 7.7</td>
</tr>
<tr>
<td>Empty Lip**</td>
<td>0.22 ± 0.01</td>
<td>40.4 ± 3.1</td>
<td>196.3 ± 1.7</td>
</tr>
<tr>
<td>Lip+VR-E2E+CpG OND**</td>
<td>0.42 ± 0.01</td>
<td>18.1 ± 2.8</td>
<td>237.9 ± 1.1</td>
</tr>
</tbody>
</table>

Values represent means ± SD (n = 3).

*the characterization of fresh Liposome solution

** Liposomes in dissolving PVP microneedle

2.2 Fabrication and characterization of DMA

The microneedle master-molds were made of aluminum with 36 (6×6) microneedles over an area of 6 ×6 mm². A PDMS mold containing inverted wells was fabricated by pouring DMS over the microneedle master-mold and incubating it at 80 °C for 1 h to make the polymer cure. The dissolving microneedles were prepared as follows: a solution for forming the tip of microneedles (tip solution) was prepared by dissolving PVP-K17 in the solution loaded with VR-E2E, or VR-E2E +CpG ODN, or Lip+VR-E2E, or Lip+VR-E2E+CpG ODN, or Lip-pGFP. And the concentration of VR-E2E, pGFP and CpG ODN in the tip solution was 0.5 mg/ml, 0.5 mg/ml and 1 mg/ml, if any. The tip solution was applied to the PDMS mold. After the tip solution dried, a 50% (w/w) PVP-k30 solution was added to the mold and dried. Finally, the microneedle arrays were gently peeled off the mold.
Fig. 2 Photographs of DMA with Trypan Blue in microneedle tips. (A) Overhead view of DMA by digital camera; (B) dark-field micrograph of a single microneedle.

To investigate the localization of vaccine, we used trypan blue as the model drug loaded only in the microneedle tips. The micrographs of DMA containing Trypan Blue were shown in Fig. 2. Fig. 2A showed a 6×6 microneedle array containing trypan blue only in the microneedle tips. And Fig. 2B showed a typical microneedle, with a length of about 650 μm, and the mean length of the drug-loaded space was about 470 μm.

2.3 Delivery of plasmid DNA to mouse skin using DMA

For effective DNA vaccination, delivered DNA should transfect skin cells. Therefore, we first tested delivery of the reporter plasmid DNA expressing green fluorescent protein. Mice were treated with DMAs loaded with or without Lip+pGFP. The treated skin area was stripped from the mice at 0, 1, 4, 7 days, and immediately imaged using a multiphoton confocal microscopes (Nikon, A1RMP). The excitation wavelength was 405 nm and 488 nm. The skin was imaged from the stratum corneum side and up to the depth of about 200 μm. 3D graphs of the visualized area were shown in Fig. 3. The negative control showed similar images at each point of time, so only one image was shown (Fig. 3A) that there were regular blue fluorescent columns through the skin. The blue fluorescent columns were also shown in other time point of images (Fig. 3B-D), which were suggested to be the autofluorescence of mouse hair. From day 0 to day 4, the GFP level was increasing. The highest GFP level was observed at day 4 (Fig.3C), almost the entire skin area was green. And at day 7, the GFP expression level in the skin significantly decreased. Yan et al. had reported a DNA delivery method with a motorized microneedle device8. They observed the highest gene expression level in the skin at day 1, and after that, the gene expression level decreased over the time quickly. Similar transient gene expression in the skin was also observed in the delivery of DNA into rat skin with an electroporation method9 and in the delivery of DNA into ex vivo human skin with a tattooing device10. The long duration of gene expression in this study might due to the sustained release of cationic liposomes11.
Fig. 3 Expression of pGFP in skin after DMA application. (A) negative control without pGFP loaded; (B) 1 day after Lip+pGFP loaded DMA application; (C) 4 days after Lip+pGFP loaded DMA application; (D) 7 days after Lip+pGFP loaded DMA application.

2.4 Immune response

In order to investigate the immune response, female Balb/c mice (six in each group) were vaccinated. Before microneedle treatment, the hair of abdominal skin was carefully shaved using electric clippers and rested for 24h. DMAs were inserted into the abdominal skin of mice for 3 min. This procedure was repeated again after 3 weeks. All mice were bled from the tail vein 2 weeks after final vaccination, and the serum was separated for antibody titers analysis. The anti-HBsAg antibody titer in serum was measured by ELISA as previously reported.

We first investigate the influence of adjuvant and delivery routes to the immune response of VR-E2E. The immune groups were as follows. 1) DMA containing only VR-E2E; 2) DMA containing VR-E2E and CpG ODN; 3) DMA containing Lip+VR-E2E; 4) DMA containing Lip+VR-E2E+CpG ODN; 5) Intramuscular (IM) immunization with Lip+VR-E2E; 6) IM with Lip+VR-E2E+CpG ODN; 7) DMA containing Lip+CpG ODN. Among these groups, Group 5) and 6) were positive control, Group 7) was negative control. VR-E2E and CpG ODN were both 10 μg/dose, if any.

Fig. 4 showed the antibody response generated by different groups. It was shown that using Lip+VR-E2E or Lip+VR-E2E+CpG ODN, DMA vaccination induced similar anti-HBsAg IgG titers with IM immunization (P>0.05). It might due to two reasons: 1) during the DMA preparation, a fraction of VR-E2E was diffused from the tip to the base; 2) during the DMA insertion, although the microneedles were totally dissolved in 3 min (data not shown), part of DMA might be left on the skin around the microchannels. Actually, we have used Interferon α-2b as the model drug, and found the proportion of the total dose retained in needle tips was 52.9 ± 4.2 %, and the proportion delivered into skin was 31.2 ± 1.9 %, respectively. Therefore comparing to total dose entered in body in IM immunization, only a fraction of the dose loaded in DMA could be delivered into body. It implied that inducing the same immune response, the actual dose delivered into body by DMA was less than that by IM. We are currently investigating to decrease the diffusion during DMA preparation and reduce the loss during DMA insertion, and improve the efficiency of the vaccine.

In the TCI groups, the group immunized with free VR-E2E showed the lowest IgG titer. Cationic liposomes or CpG ODN alone showed similar adjuvant effect. The level of IgG in the group immunized with DMA containing Lip+VR-E2E+CpG ODN was the highest IgG titer compared with other groups (P<0.05). It suggested adjuvant effect of cationic liposomes and CpG ODN could be mutually reinforcing by this delivery method. It was consistent with previous reports that the adjuvant activity of CpG ODNs was improved by co-encapsulating them in liposome vesicles.
3 Conclusions

In this study, we showed for the first time that dissolving microneedle arrays (DMA) could be prepared for transcutaneous immunization of hepatitis B virus DNA vaccine. The encapsulation of DNA vaccine into cationic liposomes obtained a sustained release and subsequent long duration of gene expression in skin. The in vivo immune test showed effective immune response by this delivery method, and the adjuvant could improve immunogenicity. Overall, dissolving microneedle arrays present beneficial delivery method to the TCI of HBV DNA vaccine. And the adjuvant cationic liposomes and CpG ODN provides a promising immune response for the TCI of HBV DNA vaccine.

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References

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