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Intradermal delivery of nanoparticulate vaccines using coated and hollow microneedles

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Chapter 7

Summarizing discussion and prospects

Introduction

Microneedles have been extensively investigated for intradermal delivery of vaccines during the last two decades. They are, as the name suggests, needle-like structures with a length shorter than 1 mm. The microneedles can be used to effectively pierce stratum corneum, which is the upper-most layer and the main barrier of skin, thereby facilitating the delivery of vaccines into the skin [1, 2]. As the microneedles do not reach nerves and blood vessels, the application of microneedles is minimally invasive and pain free. This will minimize the stress caused by traditional hypodermic needles and thus may improve the compliance of vaccinees. Furthermore, as the skin contains a large number of antigen-presenting cells (APCs), such as epidermal Langerhans cells and dermal dendritic cells, microneedle-mediated intradermal delivery of vaccines has high potential for effective vaccination [3].

Different types of microneedles have been developed for vaccine delivery. Initially, solid microneedles were used to pierce the skin, after which vaccine formulations were applied topically onto the penetrated skin after removal of the microneedles [4]. However, an important drawback of this method is that the delivery efficiency of vaccines is low as the diffusion through the conduits is limited because of the small diameter of the conduits. To increase the delivery efficiency, coated, dissolvable and hollow microneedles are now being investigated. Coated microneedles are prepared from solid microneedle arrays by coating the microneedle surface with vaccines. After the microneedle arrays are inserted, the coating is deposited in the skin. Dissolvable microneedles are made of soluble polymers, biodegradable polymers or sugars and the vaccines are loaded in the matrix of microneedles. After insertion of microneedles in the skin, the matrix starts to dissolve or degrade, thereby releasing the vaccine. Hollow microneedles contain a conduit through which the vaccine formulation can be injected into the skin. The preparation methods of these different types of microneedles have been extensively reviewed [1-3, 5].

Nanoparticulate vaccines are antigen loaded nanoparticles with a diameter less than 1000 nm [6]. Nanoparticles are capable of protecting the antigen from degradation and increasing the uptake of antigen by APCs. Additionally, nanoparticles allow the co-delivery of antigen and adjuvant, which has been reported to be crucial for improving immune responses [7, 8]. Besides, it has been reported that the immune responses can be modified or enhanced by tuning the physicochemical characteristics of nanoparticulate vaccines, such as size, surface charge and release kinetics of antigen and adjuvant [9, 10]. Various types of nanoparticles have been investigated as vaccine delivery systems, such as polymeric nanoparticles, liposomes, inorganic nanoparticles, et al [6].

Nowadays, an increasing number of studies are focusing on the use of microneedles for intradermal delivery of nanoparticulate vaccines, aiming to combine the advantages of both microneedles and nanoparticulate vaccines. A previous study showed that microneedles coated with antigen loaded lipid nanocapsules resulted in a higher IgG2a response than plain antigen coated microneedles [11]. Other studies showed that dissolvable microneedles loaded with antigen containing PLGA nanoparticles induced stronger Th1/CD8⁺ responses than antigen solution [12, 13]. Besides, hollow microneedle injected toxoid-loaded chitosan nanoparticles induced a higher IgG2a response and stronger expression of Th1 cytokines than a commercial vaccine of tetanus toxoid [14]. All of these studies showed the advantages of using microneedles for intradermal delivery of nanoparticulate vaccines.

The aim of this thesis was to determine 1) whether microneedles can be used and optimized to effectively deliver nanoparticulate vaccines intradermally and 2) whether the physicochemical characteristics of nanoparticulate vaccines have an effect on the immunogenicity of antigens

after microneedle-mediated intradermal vaccination. In this thesis, we focused on the use of coated and hollow microneedles for the delivery of nanoparticulate vaccines. In case of coated microneedles, silicon microneedle arrays were modified with pyridine groups to obtain a pH-sensitive surface. These microneedles are capable of binding negatively charged antigens at acidic conditions and releasing the coating at neutral pH [15, 16]. In case of hollow microneedles, the microneedles were prepared by etching fused silica capillaries with hydrofluoric acid [17]. These microneedles can be used to inject liquid formulations intradermally into the skin. Microscopy images of the microneedle array and hollow microneedle are shown in **Fig. 1**.

In the studies described in this thesis, the coated and hollow microneedles were developed and combined with nanoparticles with various physicochemical characteristics. The efficacy of these antigen loaded nanoparticles with and without co-encapsulation of an immune modulator on improving or modulating the immune responses was investigated.

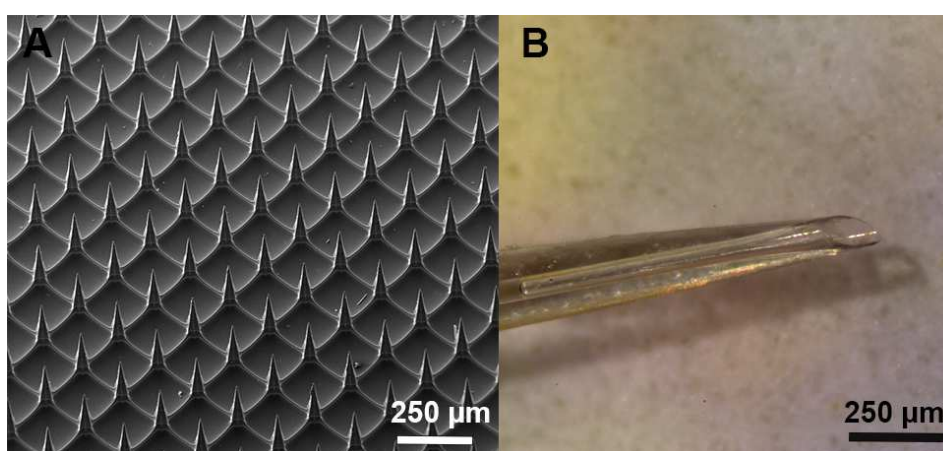


Figure 1. Microscopy images of microneedles. A: scanning electron microscopy (SEM) image of a pH-sensitive microneedle array, B: bright-field microscopy image of a hollow microneedle.

Summary of the results

In Chapter 1, a short introduction to the use of microneedles and nanoparticles for vaccine delivery via the skin is given. Next, the progress of using microneedles for intradermal delivery of nanoparticulate vaccines is briefly described. Finally, the aim and the outline of the thesis are provided.

In Chapter 2, a study is described in which a new type of mesoporous silica nanoparticles (MSNs) with large pores (about 10 nm) was successfully developed. The synthesized nanoparticles showed an efficient loading of ovalbumin (OVA) with a maximum loading capacity of about 34%. The colloidal stability of the MSNs was enhanced by coating the surface of antigen loaded MSNs with a negatively charged lipid bilayer (LB-MSN-OVA). To examine whether the MSNs could enhance antigen uptake by dendritic cells, the uptake of OVA loaded in LB-MSN-OVA by bone marrow derived dendritic cells (BMDCs) was examined. Indeed, LB-MSN-OVA showed a higher uptake than free OVA. In the next step, nanoparticles were coated onto pyridine-modified microneedle arrays. The coating process was based on the electrostatic interaction between the positively charged microneedles and the negatively charged nanoparticles. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) showed that LB-MSN-OVA were successfully coated onto the microneedle surface. About 1.5 µg of encapsulated OVA was coated onto one microneedle

array. Finally, a release study showed that LB-MSN-OVA were successfully released from the microneedles upon piercing *ex vivo* human skin.

The studies described in Chapter 3 focus on poly(lactic-co-glycolic acid) (PLGA) nanoparticles. OVA loaded PLGA nanoparticles with a positive or negative zeta potential and OVA and poly(I:C) co-encapsulated PLGA nanoparticles (with a negative zeta potential) were prepared. The effect of encapsulation of OVA with or without poly(I:C) on T cell responses was investigated after hollow microneedle mediated intradermal immunization in mice. Firstly, the capacity of OVA-loaded PLGA nanoparticles to induce T cell responses in OVA-specific T cell (OT-I and OT-II cells) transferred mice was studied. It was shown that OVA-loaded PLGA nanoparticles with a positive or negative zeta potential induced similar proliferations of the adoptively transferred OVA-specific CD8⁺ and CD4⁺ T cells, which were both significantly higher than those induced by OVA solution. Next, the capacity of PLGA nanoparticles loaded with OVA with and without poly(I:C) to induce endogenous T cell responses in wild type mice was studied. The OVA-loaded PLGA nanoparticles (both cationic and anionic), and OVA and poly(I:C) co-encapsulated nanoparticles were found to be able to induce endogenous OVA-specific CD8⁺ T cell responses in the wild type mice. The addition of poly(I:C) (either mixed with OVA solution or co-encapsulated with OVA in PLGA nanoparticles) enhanced CD8⁺ T cell responses. Furthermore, OVA loaded PLGA nanoparticles with a positive zeta potential induced stronger endogenous CD8⁺ T cell responses than anionic PLGA nanoparticles. Finally, it was studied whether the elicited endogenous T cell responses were able to protect the wild type mice from infection with OVA-secreting intracellular bacterium *Listeria monocytogenes*. It was shown that OVA and poly(I:C) co-encapsulated PLGA nanoparticles provided a full protection against the bacterium. In summary, in this study it was shown that PLGA nanoparticle formulations are excellent systems for delivery of protein antigen into the skin to induce protective cellular immune responses by using hollow microneedles for intradermal immunization.

The results of Chapter 2 and 3 demonstrate the advantages of using nanoparticulate vaccines for improving the immunogenicity of antigen. In Chapter 4, studies are reported in which hollow microneedles were used to further investigate the effect of nano-encapsulation of antigen and adjuvant on the immune responses. OVA and poly(I:C) loaded PLGA nanoparticles, liposomes, MSNs and gelatin nanoparticles (GNPs), covering a broad range of physicochemical particle characteristics, were compared. PLGA nanoparticles and liposomes showed a smaller particle size (below 200 nm) and slower release of OVA/poly(I:C) than MSNs and GNPs (mean particle size of both particles was about 500-700 nm). The vaccination studies revealed that the encapsulation of OVA and co-encapsulation of OVA and poly(I:C) in the various types of nanoparticles induced similar total IgG and IgG1 responses, but higher IgG2a antibody responses as compared to OVA/poly(I:C) solution. The type of nanoparticles had a major effect on the IgG2a response: PLGA nanoparticles and cationic liposomes induced higher responses than MSNs and GNPs, correlating with a smaller nanoparticle size and a slower release of antigen and adjuvant. When studying cell mediated immune responses, the antigen and adjuvant loaded cationic liposomes induced the strongest proliferation of adoptively transferred CD8⁺ and CD4⁺ T cells, suggesting their superiority for intradermal vaccination over the other nanoparticles. The studies described in this chapter demonstrate that the in-house developed hollow microneedles can be used to screen different nanoparticulate vaccine formulations for intradermal vaccination.

After the observation of the superior immune responses of antigen and adjuvant co-encapsulated liposomes as compared to other nanoparticles, the aim of the next series of studies was to examine whether the co-encapsulation of antigen and adjuvant in liposomes is

a crucial factor for the higher IgG2a responses. These studies have been described in Chapter 5, using the same type of cationic liposomes as in the studies reported in Chapter 4. Diphtheria toxoid (DT) and poly(I:C) were used as a model antigen and adjuvant, respectively. DT and poly(I:C) were either individually encapsulated or co-encapsulated in the liposomes with high loading efficiencies of more than 90%. After hollow microneedle-mediated intradermal immunization, the antigen- and adjuvant-containing liposomes evoked potent antibody responses and shifted the IgG1/IgG2a balance to a IgG2a response, no matter whether DT and poly(I:C) were individually encapsulated or co-encapsulated in the liposomes. These results indicate that the combination of DT and poly(I:C) individually encapsulated liposomes are as efficient as DT and poly(I:C) co-encapsulated liposomes for the modulation of the immune response.

In Chapter 6 studies are reported in which DT loaded and lipid bilayer coated MSNs (LB-MSN-DT) were coated onto pH-sensitive microneedle arrays. Additionally, the antibody response elicited by coated microneedles was compared to that elicited by hollow microneedle-delivered LB-MSN-DT. By using the same preparation method as described for OVA loaded MSNs in Chapter 2, DT was successfully loaded into MSNs followed by fusion of a negatively charged lipid bilayer onto the surface of MSNs. The synthesized nanoparticles showed a high loading capacity of DT of about 20%. The LB-MSN-DT and N-trimethyl chitosan (TMC) were alternately coated onto the pH sensitive surface of the pyridine-modified microneedle arrays by using a layer-by-layer coating approach. SEM and CLSM images demonstrated that LB-MSN-DT were successfully coated onto the microneedle surfaces. It was shown that the cumulative coated amount of nano-encapsulated DT for a 5-layer and 3-layer coating on the microneedle surface of one microneedle array was about 1.9 μg and 1.1 μg , respectively, corresponding to 9.7 μg and 5.7 μg LB-MSN-DT (expressed as the weight of MSNs). A release study in *ex vivo* human skin showed that 0.814 μg and 0.256 μg of the encapsulated DT were released from a 5-layer and 3-layer coated microneedle array, respectively. An *in vivo* study in mice showed that LB-MSN-DT delivered by both coated and hollow microneedles successfully induced DT-specific antibody responses. The nano-encapsulated DT induced stronger antibody responses than DT solution when delivered by hollow microneedles (after 1st boost immunization), but induced only comparable responses as DT solution when delivered by coated microneedles. The results of the research described in this chapter demonstrate that both the encapsulation of antigen and the type of microneedles can affect the immunogenicity of antigen, and that the coated microneedle system may need to be improved in order to obtain optimal immune responses.

In summary, the collective results described in this thesis show that nanoparticulate vaccines can be delivered intradermally by coated and hollow microneedles and evoke antigen-specific immune responses. The choice of both the nanoparticles and the microneedle(s) could have important influences on the immune responses. Microneedle arrays coated with antigen loaded and lipid bilayer fused MSNs could be a promising system for convenient and fast intradermal delivery of protein antigen, although our results indicate that the system needs to be improved in order to obtain optimal immune responses. Moreover, antigen and adjuvant loaded nanoparticles can increase IgG2a (Th1) and CD8⁺ responses after intradermal delivery by hollow microneedles. This effect depends on the type and the physicochemical characteristics of the nanoparticles, in which smaller size and controlled release properties of antigen and adjuvant were found to correlate with the stronger effect. Finally, the combination of separate antigen loaded and adjuvant loaded nanoparticles may be as efficient as the antigen and adjuvant co-encapsulated nanoparticles for modification of the immune responses following intradermal immunization.

Discussion and prospects

MSN coated microneedle arrays for intradermal delivery of protein antigen

One main goal of the work described in this thesis was to optimize coated microneedles for intradermal delivery of nanoparticulate vaccines. For coated microneedles, one limiting factor is the relatively small coating amount of antigen due to the small surface area of microneedles. In the studies described in this thesis, MSNs with large pores (10 nm in diameter) were synthesized to allow for efficient loading of antigens. Indeed, it was shown that the synthesized MSNs had a high loading capacity of OVA and DT, which was about 20% for both antigens. The high loading capacity of antigens in MSNs together with the strong surface charge of MSNs may synergistically have led to the higher coating amounts of antigen onto the microneedle array surfaces (about 500 ng per layer) as compared to our previous study (about 300 ng per layer), in which plain DT was coated onto the same type of microneedle arrays [18]. Previously, a layer-by-layer coating approach was used for the coating of antigen onto the pH-sensitive microneedle arrays, in which the coating amount of antigen could be tailored by adjusting the number of coating layers [18, 19]. In Chapter 6 it was shown that this multilayer coating method can also be used for the coating of antigen loaded MSNs.

Besides an adequate coating amount of antigen, it is important that the coated antigen can be released fast into the skin. We showed that by using a multiple insertion mode (10 times penetration in 10 s) of the microneedles, the release efficiency of the coated antigen was significantly increased and the required wearing time of the microneedles was significantly decreased. The shorter wearing time of microneedles may help improving the compliance of vaccinees. However, a disadvantage of using the multiple insertion mode is that an expensive applicator needs to be used. If the applicator is put onto the medical market in the future, the scale production may help decreasing the cost per applicator.

The immunization studies in mice showed that DT encapsulated MSNs induced stronger immune responses than DT solution when delivered by hollow microneedles, but only induced comparable responses as DT solution when delivered by coated microneedles. The results of coated microneedles are contradictory to the results reported in a previous study, in which microneedles coated with OVA loaded lipid nanocapsules enhanced immune responses compared to those induced by plain OVA coated microneedles [11]. In that study, the lipid nanocapsules and a hydrolytically degradable poly(β -amino ester) were alternately coated onto microneedles by using a layer-by-layer coating approach. The results showed that the multilayers were successfully released into the skin and completely broke down within 24 h, thereby allowing uptake of the nanocapsules by APCs. A possible reason for the lower responses of the coated microneedles in the study described in Chapter 6 is that the individual LB-MSN-DT nanoparticles cannot escape from the multiple nanoparticle/TMC layer deposited in the skin. As a result, the nanoparticles may not be efficiently taken up by APCs. It would therefore be interesting to study the use of polymers which are easier to degrade (for example, poly(β -amino ester)) or less viscous (for example, TMC with a lower molecular weight).

Hollow microneedle mediated intradermal delivery of nanoparticulate vaccines

In the studies described in this thesis, hollow microneedles are used as a tool to investigate the effect of the physicochemical characteristics of nanoparticulate vaccines on the immune responses. Previously, it has been shown that antigen and immune modulator co-encapsulated nanoparticles were able to enhance IgG2a and CD8⁺ T cell responses after traditional hypodermic needle mediated intradermal vaccination [8, 20, 21]. Our results showed that this

trend remains in hollow microneedle-mediated intradermal vaccination. The results showed that the co-delivery of antigen and adjuvant by using nanoparticles significantly increased IgG2a titers. Co-encapsulation of antigen and adjuvant (poly(I:C)) may allow the delivery of antigen and adjuvant in the same antigen presenting cells, which may increase the presentation of antigen to T cells [20, 22]. Furthermore, nanoparticles with a smaller size and slower release of antigen and adjuvant induced stronger IgG2a titers. The smaller size of nanoparticles may enhance the uptake of antigen and adjuvant by antigen presenting cells [9, 23]. These results demonstrate that the quality and type of immune responses can be modified to desired direction by using nanoparticles with appropriate physicochemical properties.

The results described in the thesis further showed that individual encapsulation of antigen and adjuvant is as efficient as co-encapsulation of antigen and adjuvant in liposomes for the induction of higher IgG2a titers, indicating that co-encapsulation of antigen and adjuvant may not be necessary for the use of intradermal delivery. This might help simplifying the work for development of liposomal formulations. The formulations can be made by simply mixing antigen loaded liposomes and adjuvant loaded liposomes. It will be interesting to test whether these findings remain when other types of TLR ligands are used. Overall, the results described in the thesis indicate that the optimal nanoparticles for intradermal use should be encapsulated with antigen and adjuvant (either individually encapsulated or co-encapsulated), have a small particle size (below 200 nm) and a sustained release of antigen and adjuvant.

In the research described in this thesis, it was also shown that co-encapsulation of antigen and adjuvant in 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP)-based cationic liposomes induced potent Th1/CD8⁺ T cell responses. Furthermore, the liposomes induced the strongest T cell responses among four types of the nanoparticles. Some recent studies also reported that peptide-loaded cationic liposomes were able to induce effective T cell responses for the prevention of tumor growth [24] and clearance of established tumors [25]. These results demonstrate the potential of cationic liposomes for inducing high T-cell responses, which are important for treatment of tumors and combat against intracellular bacteria. In the future, it would be important to test whether the effectiveness of cationic liposome formulations holds true for other tumor models.

Prospects of the use of microneedles for intradermal delivery of nanoparticulate vaccines

In case of hollow microneedles, the antigen and adjuvant loaded nanoparticles are suspended in buffer before the injection and the physicochemical properties of the nanoparticulate vaccines probably do not change during the injection by hollow microneedles. As a result, by using hollow microneedles it is convenient to study the effect of the physicochemical characteristics of nanoparticles on the immune responses. Furthermore, by using hollow microneedles, the influence of injection depth on the immune responses can be easily studied [26]. Instead, in case of coated and dissolvable microneedles, the nanoparticles suspended in buffer are first coated onto or loaded into the microneedles. The nanoparticles are dried during the fabrication of microneedles and released from the microneedles after the penetration of the skin. During this process, it is possible that the properties of nanoparticles and the encapsulated antigen are impacted. For example, the nanoparticles may aggregate and the antigen may lose some activity. Therefore, hollow microneedles may be more suitable for preliminary research, such as screening of nanoparticulate vaccines for intradermal vaccination and study of the effect of physicochemical characteristics of nanoparticles on immune responses. After the screening, the selected nanoparticulate vaccines can be used for the development of coated or dissolvable microneedles.

Chapter 7

Although the results in this thesis showed that the coated and hollow microneedles can be used to intradermally deliver the nanoparticulate vaccines and evoke antigen specific immune responses, further research is needed to develop and optimize the two technologies. The interest in combining microneedle and nanoparticulate vaccine technologies will continue to grow with the emergence of new types of nanoparticles and fabrication methods of microneedles. Finally, the largest challenge is to translate the research to products which can finally benefit patients. This will need continuous and joint efforts from academia and the pharmaceutical industry.

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