

## SHORT COMMUNICATION

# Cutaneous vaccination using microneedles coated with hepatitis C DNA vaccine

HS Gill<sup>1,4</sup>, J Söderholm<sup>2,5</sup>, MR Prausnitz<sup>1,3</sup> and M Sällberg<sup>2</sup>

<sup>1</sup>Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA, USA; <sup>2</sup>Division of Clinical Microbiology, F68, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden; <sup>3</sup>School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, USA

*The skin is potentially an excellent organ for vaccine delivery because of accessibility and the presence of immune cells. However, no simple and inexpensive cutaneous vaccination method is available. Micron-scale needles coated with DNA were tested as a simple, inexpensive device for skin delivery. Vaccination with a plasmid encoding hepatitis C virus nonstructural 3/4A protein using microneedles effectively*

*primed specific cytotoxic T lymphocytes (CTLs). Importantly, the minimally invasive microneedles were as efficient in priming CTLs as more complicated or invasive delivery techniques, such as gene gun and hypodermic needles. Thus, microneedles may offer a promising technology for DNA vaccination. Gene Therapy (2010) 17, 811–814; doi:10.1038/gt.2010.22; published online 4 March 2010*

**Keywords:** cellular immune response; DNA-coated microneedle; DNA vaccine; hepatitis C virus; microfabricated needle

## Introduction

DNA vaccines are relatively simple to produce and have the potential to generate strong cellular and humoral immune responses, making them attractive vaccine candidates.<sup>1</sup> However, a major shortcoming of DNA vaccines is their poor immunogenicity when administered intramuscularly, characteristically requiring at least 5–10 mg of DNA to generate a robust immune response in humans.<sup>2</sup> Cutaneous immunization using gene gun or electroporation has improved potency by approximately three orders of magnitude in humans and nonhuman primates.<sup>2</sup> However, gene gun and electroporation require elaborate vaccination protocols and equipment.<sup>3</sup> Therefore, there is a need for a convenient and low-cost cutaneous DNA vaccine delivery method that can generate robust cellular responses.

Recently, microneedles have been developed by adapting the technology of microelectronics industry to fabricate micron-scale needles that painlessly deliver compounds into the skin, using methods suitable for inexpensive mass production.<sup>4</sup> In one approach, solid microneedles have been coated with drugs or vaccines as a solid film on the microneedle surface. After insertion into the skin, cutaneous interstitial fluid rapidly

dissolves the film and releases drug or vaccine into the skin. Coated microneedles have been used previously to vaccinate mice with inactivated influenza virus and guinea pigs with ovalbumin.<sup>5,6</sup>

Using a related approach, scraping blunt-tipped microneedles on the skin after topical application of a solution of plasmid DNA has been used to vaccinate mice.<sup>7</sup> Using another approach, hollow microneedles have been used to inject antigens into the skin of animals and humans to generate humoral responses to a number of antigens.<sup>4,8</sup> Microneedles have also been combined with electroporation to induce humoral responses in nonhuman primates using a smallpox DNA vaccine.<sup>9</sup>

Among the various approaches, coated microneedles are especially attractive as a method for rapid administration of vaccines with little or no training because microneedles are precoated with vaccines that can be dissolved in the skin's interstitial fluid within seconds and can be prepared as adhesive patch-like devices for self-application.<sup>10</sup> Robust cytotoxic T lymphocyte (CTL) activity is an important component of cellular immunity responsible for killing pathogen-infected cells and clearing the pathogen. Previous studies have not assessed CTL activity after microneedle vaccination. In this study, the hypothesis that DNA-coated microneedles can elicit a robust CTL cellular immune response was tested.

This hypothesis was tested using a well-characterized DNA vaccine encoding hepatitis C virus nonstructural (NS) 3/4A protein that has previously been shown to induce strong *in vivo* functional T-cell responses in mice when delivered by gene gun or intramuscular injection, followed by *in vivo* electroporation.<sup>11,12</sup> In this study, cutaneous DNA delivery using microneedles was compared with (1) intramuscular DNA delivery by hypodermic injection, which is widely used in animal studies but is generally ineffective in humans and (2) cutaneous

Correspondence: Dr M Sällberg, Division of Clinical Virology, F68, Huddinge University Hospital, S-141 86 Stockholm, Sweden.

E-mail: matti.sallberg@ki.se or Dr MR Prausnitz, School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, 311 Ferst Drive, Atlanta, GA 30332-0100 USA.

E-mail: prausnitz@gatech.edu

<sup>4</sup>Current address: Department of Chemical Engineering, Texas Tech University, 6th and Canton, Lubbock, TX 79409, USA.

<sup>5</sup>Current address: Department of Infectious Diseases, University of Gothenburg, Gothenburg, Sweden.

Received 13 April 2009; revised 6 December 2009; accepted 7 December 2009; published online 4 March 2010

delivery using gene gun, which can be effective in humans.<sup>13</sup>

## Results and Discussion

### Plasmid-coated microneedles

Rows of microneedles were designed to enable consistent penetration into the highly pliant skin of mice. Each microneedle row contained five microneedles measuring 700  $\mu\text{m}$  in length and 160  $\mu\text{m}$  in width at the base, which tapered to a sharp tip with  $<1\text{ }\mu\text{m}$  radius of curvature (Figure 1a). A previously developed dip-coating method was used to achieve uniform plasmid films on microneedles.<sup>10</sup> The coating formulation and process are described in Figure 1. Owing to difficulty in visualizing plasmid coatings, microneedles were alternatively coated with vitamin B<sub>2</sub> as a model, colored compound to visually assess coating uniformity. After coating with vitamin B<sub>2</sub>, all five microneedles of the row were found to be uniformly coated (Figure 1b). Subsequently, fresh microneedles were coated with DNA, and each microneedle row was found to be coated with  $1.6 \pm 0.2\text{ }\mu\text{g}$  of DNA.

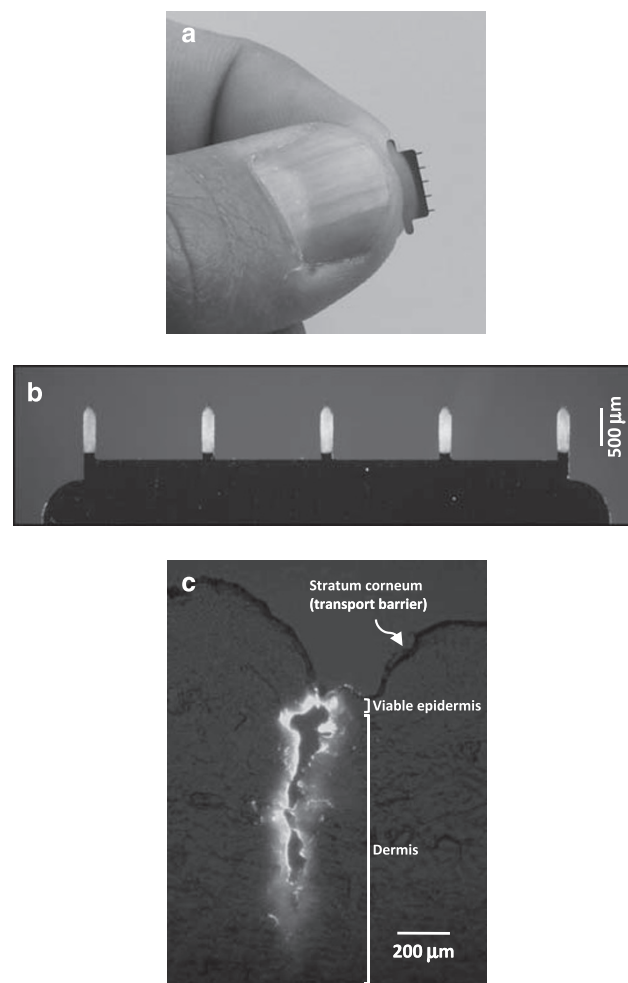
Microneedles were designed with a length of 700  $\mu\text{m}$  for intracutaneous delivery to the human skin, which has a representative thickness of 2–3 mm. Histological evaluation of the skin after piercing it with sulforhodamine-coated microneedles demonstrates that microneedles deposited the coating in pig skin, which is a good model of human skin anatomy (Figure 1c). Although the mouse skin is approximately three times thinner, microneedles are expected to remain within the skin of mice too.

This result is consistent with previous work in which microneedles were uniformly coated with fluorescently labeled plasmid DNA.<sup>10</sup> Furthermore, coated microneedles have been shown to have  $\leq 90\%$  delivery efficiency.<sup>10</sup> Accordingly,  $1.4\text{ }\mu\text{g}$  of the total  $1.6 \pm 0.2\text{ }\mu\text{g}$  DNA coated on microneedles is expected to be delivered into the skin.

### Priming of NS3-specific CTLs by microneedle delivery

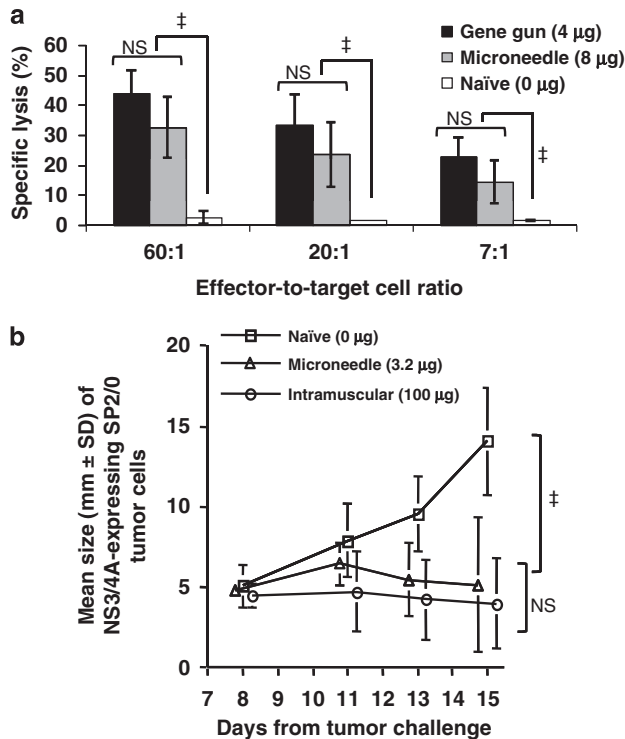
To determine whether cutaneous immunization of mice with microneedles coated with plasmid encoding hepatitis C virus NS3/4A protein could elicit a lytic cellular immune response, mice were vaccinated with 8  $\mu\text{g}$  DNA using microneedles and 4  $\mu\text{g}$  DNA using gene gun. Indeed, immunization with DNA-coated microneedles induced the generation of lytic CTLs (Figure 2a). At each effector-to-target ratio, cell lysis was significantly higher after microneedle treatment compared with naive mice ( $P < 0.05$ ). This indicates that microneedles released coated DNA that primed, most likely in the skin, a cellular immune response.

The level of CTLs primed by microneedle-based immunization was found to be comparable with that observed with gene gun-based immunization ( $P > 0.05$ , Figure 2a). A significant target-cell lysis was observed even at low effector-to-target ratios, indicating a robust immune response from both the DNA-coated microneedle and gene gun-based immunizations. These results are consistent with previous cutaneous immunization studies using a gene gun, in which it was found that gene gun-based cutaneous delivery of low ( $\leq 10\text{ }\mu\text{g}$ ) doses of coNS3/4A DNA plasmid effectively primed NS3-specific CTLs in Balb/c and C57BL/6 mice.<sup>14</sup> Although the intramuscular immunization group was not included in



**Figure 1** Coated microneedles. Microneedles were cut from stainless steel sheets using an infrared laser and electropolished as described before.<sup>10</sup> (a) Photograph of a representative microneedle row held in a human hand. (b) Microneedle row with five microneedles uniformly coated with vitamin B<sub>2</sub> as a model compound. As plasmid coatings were difficult to visualize, the uniformity of coatings was assessed by coating microneedles with a model colored compound, vitamin B<sub>2</sub> (riboflavin-5'-phosphate sodium salt dihydrate) (Fisher Scientific, Fair Lawn, NJ, USA). The aqueous dip-coating solution contained 1% (w/v) carboxymethylcellulose sodium salt (CMC, low viscosity, USP grade, CarboMer, San Diego, CA, USA), 0.5% (w/v) Lutrol F-68 NF (BASF, Mt Olive, NJ, USA) and 20 mg ml<sup>-1</sup> vitamin B<sub>2</sub>.<sup>18</sup> Microneedles were coated using a custom dip-coating device and air dried for  $>24\text{ h}$ .<sup>10</sup> For immunization, microneedles were coated using the same formulation, but by replacing vitamin B<sub>2</sub> with 5 mg ml<sup>-1</sup> codon-optimized NS3/4A plasmid DNA. To determine the amount of DNA coated on microneedles, DNA concentration was measured by a validated technique using ultraviolet (UV) absorbance at 260 nm in a solution prepared by vortexing coated microneedles for 1 min in 1 ml deionized water. (c) Histological section of porcine cadaver skin after inserting sulforhodamine-coated microneedle. To histologically characterize insertion of microneedles into the skin, microneedles were coated with 0.1% (w/v) sulforhodamine (Molecular Probes, Eugene, OR, USA) with CMC and Lutrol F-68 NF, as described above, and inserted into porcine cadaver skin for 1 min.

this experiment, our previous data showed that a single intramuscular injection at a similar dose and without any adjuvant, fails to effectively induce significant CTL activity.<sup>12</sup> These results indicate that, similar to the gene gun, microneedle-based cutaneous immunization in



**Figure 2** Microneedle-based immunization induces antigen-specific CTLs. Groups of 4–8-week-old female C57BL/6 or Balb/c mice (Charles River, Uppsala, Sweden) were immunized cutaneously with microneedles or gene gun, or intramuscularly using hypodermic needles. For microneedle-based immunization, two or five rows per mouse were used to control the dose. Each microneedle row was coated with 1.6 µg DNA. DNA-coated microneedle rows were manually inserted into the trimmed abdominal or back skin and held for 1 min to allow dissolution of coated DNA into the skin. For gene gun-based immunization, mice were immunized by gene gun (Bio-Rad Laboratories, Hercules, CA, USA) at a dose of 4 µg per mouse as described previously.<sup>12</sup> Plasmid DNA was linked to 1-µm diameter gold particles for gene gun-based immunizations according to protocols supplied by the manufacturer. For intramuscular immunization, mice were injected with 100 µg DNA in the tibialis anterior using a hypodermic needle. Untreated (naïve) mice were used as a negative control. All experimental protocols were approved by the ethics committee for animal research at the Karolinska Institutet (Stockholm, Sweden). (a) C57BL/6 mice immunized once with codon-optimized NS3/4A DNA using either microneedles (1.6 µg per row × 5 microneedle rows per mouse = 8 µg dose;  $n=4$  mice; gray bars), gene gun (4 µg dose;  $n=4$  mice; black bars) or no immunization ( $n=2$  mice; white bars) and killed after 2 weeks. As described previously,<sup>12,14</sup> cells harvested from the spleen (effector cells,  $2.5 \times 10^7$  cells per mouse) were restimulated with a NS3 H-2D<sup>b</sup>-specific peptide and  $2.5 \times 10^7$ -irradiated naïve C57BL/6 splenocyte cells. After 5 days,  $5 \times 10^3$  RMA-S cells pulsed with NS3 H-2D<sup>b</sup>-specific peptide and labeled with  $^{51}\text{Cr}$  were used as target cells. The specific cell lysis of target cells was then measured at different effector-to-target cell ratios by measuring  $^{51}\text{Cr}$  released from lysed target cells. (b) Balb/C mice were either immunized intramuscularly (100 µg;  $n=5$  mice), cutaneously with microneedles (1.6 µg per row × 2 microneedle rows per mouse = 3.2 µg dose;  $n=5$  mice) or were not immunized ( $n=2$  mice). The presence of *in vivo* functional CTLs was determined using a tumor challenge model.<sup>14</sup> Two weeks after immunization, mice were injected subcutaneously with  $1 \times 10^6$  SP2/0 myeloma cells stably transfected with NS3/4A. Tumor growth was then monitored daily through the skin by recording the mean tumor size (thickness of skin flap at tumor injection site) for 14 days and compared with growth of the same tumor cell line in nonvaccinated mice. Mean tumor sizes were compared by analysis of variance (ANOVA,  $\alpha=0.05$ ). Error bars represent s.d.; symbol † represents  $P<0.05$ ; NS means not significant.

mice induces a robust CTL response at low microgram doses without the use of an adjuvant. However, in contrast to gene gun, microneedle delivery does not require sophisticated instrumentation.

#### In vivo functionality of primed NS3-specific CTLs

The *in vivo* functionality of primed CTLs was determined as the ability to inhibit growth of NS3/4A-expressing tumor cells *in vivo* after challenge. This assay determines whether CTLs are functional *in vivo* such that they recognize and lyse a syngeneic cell line stably transfected to express NS3/4A. This assay showed that tumors in naïve mice grew until the experiment had to be terminated to avoid undue animal suffering (Figure 2b). In contrast, tumor growth was significantly inhibited in mice treated with microneedles coated with 3.2 µg plasmid ( $P<0.05$ ). Tumor growth after intramuscular delivery of 100 µg plasmid was also inhibited relative to naïve mice ( $P<0.05$ ) and to a similar extent as microneedles ( $P>0.05$ ). Altogether, this result shows that microneedle immunization activates NS3-specific CTLs that are functional *in vivo*, and that primed CTLs can recognize, enter and eliminate an NS3/4A-expressing tumor. Importantly, microneedle-immunized mice received much less DNA compared with intramuscularly injected mice, but were equally effective. This supports our previous observations that NS3/4A-DNA effectively primes specific *in vivo* functional CTLs by low-dose cutaneous administration.<sup>12</sup> Although a gene gun-immunized group was not included in this study, our earlier experiments have repeatedly shown that cutaneous delivery of 4 µg DNA in mice using gene gun also leads to the inhibition of tumor growth.<sup>14</sup>

Overall, this study assessed the potential of DNA-coated microneedles to induce a CTL response and compared it with gene gun and intramuscular routes. Microneedles are an attractive delivery system because they have previously been shown to cause little or no pain in humans,<sup>15</sup> can be produced at disposable cost on at an industrial scale<sup>4</sup> and are expected to be easily administered by minimally trained personnel, with the possibility of self-administration. Although rows of microneedles were used in this study to facilitate penetration into the highly deformable mouse skin, two-dimensional arrays of microneedles can be easily fabricated as patches containing up to a few hundred microneedles each, allowing delivery of up to hundreds of micrograms of DNA. Noncoated microneedle patches have been applied to the skin of humans hundreds of times in our laboratory using simple manual insertion and have generated no adverse effects.<sup>15,16</sup>

Gene gun-mediated delivery has consistently shown good potency in various animal models, including larger animals and humans.<sup>13</sup> However, clinical and logistical applicability of gene gun remain uncertain. This study showed that coated microneedle- and gene gun-based cutaneous immunizations using low doses of coNS3/4A DNA (without adjuvants) resulted in comparable levels of *in vitro* and *in vivo* priming of antigen-specific CTLs. The same level of protection through intramuscular administration used a >30-fold higher dose. Previous work has shown that the intramuscular dose can be reduced if injection is administered with the help of adjuvants, such as delivery by *in vivo* electroporation.<sup>11</sup> The experiments in this study using the gene gun and



intramuscular injection control are fully consistent with previous observations.<sup>14</sup> Thus, it is our hypothesis that microneedle patches may provide a DNA vaccine delivery method with adequate efficacy that is also simple, inexpensive and apparently safe. Additional research in larger animals and humans is required to more fully validate this hypothesis.

Mechanistically, it is expected that several different cell populations may have helped to generate a potent immune response from DNA-coated microneedle-based immunization. Both Langerhans and epithelial cells of the skin may be transfected by the NS3/4A DNA, in addition to dendritic and other cells of the dermis. Thus, both direct antigen presentation and cross-presentation, as reported when using the gene gun,<sup>17</sup> could be responsible for priming the immune response.

In conclusion, this study shows that a NS3/4A-expressing DNA plasmid can be delivered into the skin using coated microneedles to elicit CTLs specific for hepatitis C virus. Importantly, CTL priming using microneedles was similar to gene gun at similar doses, which suggests that immune responses generated using microneedles may be sufficient for DNA vaccine applications.

## Conflict of interest

Authors Harvinder S Gill and Mark R Prausnitz are coinventors on a microneedle-based patent that has been licensed to a company. In addition, Mark Prausnitz is a coinventor on other patents and is a consultant and advisor to companies working on microneedles. Although there are currently no products based on microneedles, and this study does not involve any commercial products, the results of this research could indirectly influence the success of possible future commercial activities in which the authors have an interest. Authors Jonas Söderholm and Matti Sällberg have no conflict of interest.

## Acknowledgements

This study was supported by the Swedish Science Council, the Cancer Foundation, Stockholm County Council, US National Institutes of Health, and Robert M Nerem Travel Award to HSG.

## References

- Garmory HS, Perkins SD, Phillpotts RJ, Titball RW. DNA vaccines for biodefence. *Adv Drug Deliver Rev* 2005; **57**: 1343–1361.
- Donnelly J, Berry K, Ulmer JB. Technical and regulatory hurdles for DNA vaccines. *Int J Parasitol* 2003; **33**: 457–467.
- Nicolas JF, Guy B. Intradermal, epidermal and transcutaneous vaccination: from immunology to clinical practice. *Expert Rev Vaccines* 2008; **7**: 1201–1214.
- Prausnitz MR, Mikszta JA, Cormier M, Andrianov AK. Micro-needle-based vaccines. *Curr Top Microbiol Immunol* 2009; **333**: 369–393.
- Koutsouanos DG, del Pilar Martin M, Zarnitsyn VG, Sullivan SP, Compans RW, Prausnitz MR *et al*. Transdermal influenza immunization with vaccine-coated microneedle arrays. *PLoS One* 2009; **4**: e4773.
- Widera G, Johnson J, Kim L, Libiran L, Nyam K, Daddona PE *et al*. Effect of delivery parameters on immunization to ovalbumin following intracutaneous administration by a coated microneedle array patch system. *Vaccine* 2006; **24**: 1653–1664.
- Mikszta JA, Alarcon JB, Brittingham JM, Sutter DE, Pettis RJ, Harvey NG. Improved genetic immunization via micromechanical disruption of skin-barrier function and targeted epidermal delivery. *Nat Med* 2002; **8**: 415–419.
- Van Damme P, Oosterhuis-Kafeja F, Van der Wielen M, Almagor Y, Sharon O, Levin Y. Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults. *Vaccine* 2009; **27**: 454–459.
- Hooper JW, Golden JW, Ferro AM, King AD. Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge. *Vaccine* 2007; **25**: 1814–1823.
- Gill HS, Prausnitz MR. Coated microneedles for transdermal delivery. *J Control Release* 2007; **117**: 227–237.
- Ahlen G, Soderholm J, Tjelle T, Kjeker R, Frelin L, Hoglund U *et al*. *In vivo* electroporation enhances the immunogenicity of hepatitis C virus nonstructural 3/4A DNA by increased local DNA uptake, protein expression, inflammation, and infiltration of CD3+ T cells. *J Immunol* 2007; **179**: 4741–4753.
- Frelin L, Alheim M, Chen A, Soderholm J, Rozell B, Barnfield C *et al*. Low dose and gene gun immunization with a hepatitis C virus nonstructural (NS) 3 DNA-based vaccine containing NS4A inhibit NS3/4A-expressing tumors *in vivo*. *Gene Therapy* 2003; **10**: 686–699.
- Fuller DH, Loudon P, Schmaljohn C. Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. *Methods* 2006; **40**: 86–97.
- Frelin L, Ahlen G, Alheim M, Weiland O, Barnfield C, Liljestrom P *et al*. Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus non-structural 3/4A gene. *Gene Therapy* 2004; **11**: 522–533.
- Gill HS, Denson DD, Burris BA, Prausnitz MR. Effect of microneedle design on pain in human volunteers. *Clin J Pain* 2008; **24**: 585–594.
- Wermeling DP, Banks SL, Hudson DA, Gill HS, Gupta J, Prausnitz MR *et al*. Microneedles permit transdermal delivery of a skin-impermeant medication to humans. *Proc Natl Acad Sci USA* 2008; **105**: 2058–2063.
- Cho JH, Youn JW, Sung YC. Cross-priming as a predominant mechanism for inducing CD8(+) T cell responses in gene gun DNA immunization. *J Immunol* 2001; **167**: 5549–5557.
- Gill HS, Prausnitz MR. Coating formulations for microneedles. *Pharm Res* 2007; **24**: 1369–1380.