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Research Paper

Gene Delivery to the Epidermal Cells of Human Skin Explants Using Microfabricated Microneedles and Hydrogel Formulations

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Purpose. Microneedles disrupt the stratum corneum barrier layer of skin creating transient pathways for the enhanced permeation of therapeutics into viable skin regions without stimulating pain receptors or causing vascular damage. The cutaneous delivery of nucleic acids has a number of therapeutic applications; most notably genetic vaccination. Unfortunately non-viral gene expression in skin is generally inefficient and transient. This study investigated the potential for improved delivery of plasmid DNA (pDNA) in skin by combining the microneedle delivery system with sustained release pDNA hydrogel formulations.
Materials and Methods. Microneedles were fabricated by wet etching silicon in potassium hydroxide. Hydrogels based on Carbopol polymers and thermosensitive PLGA-PEG-PLGA triblock copolymers were prepared. Freshly excised human skin was used to characterise microneedle penetration (microscopy and skin water loss), gel residence in microchannels, pDNA diffusion and reporter gene (β-galactosidase) expression.
Results. Following microneedle treatment, channels of approximately 150–200 μm depth increased trans-epidermal water loss in skin. pDNA hydrogels were shown to harbour and gradually release pDNA. Following microneedle-assisted delivery of pDNA hydrogels to human skin expression of the pCMVβ reporter gene was demonstrated in the viable epidermis proximal to microchannels.
Conclusions. pDNA hydrogels can be successfully targeted to the viable epidermis to potentially provide sustained gene expression therein.

KEY WORDS: DNA; human skin; hydrogel; microneedles; thermosensitive.

INTRODUCTION

A rapidly increasing body of evidence has shown that microfabricated microneedles are effective in disrupting the primary physical barrier of the skin, the stratum corneum (SC), to affect the intra- and trans-cutaneous delivery of both low molecular weight drugs and macromolecules (1–7). As a consequence, microneedles are also being investigated as a method for delivering plasmid DNA (pDNA) to the viable epidermis of the skin with potential applications in the gene based treatment of cancers or genetic diseases, and for DNA vaccination (8,9). Cutaneous DNA vaccination is a particularly attractive proposition since immune surveillance is a primary function of the skin (10). Epidermal dendritic cells (DCs) called Langerhans cells (LCs) are primarily responsible for antigen presentation and the consequential T-cell mediated immune responses and therefore this cell population represents an attractive target for antigenic stimulation following the cellular expression of a genetic vaccine (11). Microneedles transiently create microscopic channels that penetrate through the SC and extend into the epidermis (2) but do not stimulate pain receptors that are located within the underlying dermis. Therefore microneedle delivery of medicaments and vaccines to the epidermal strata is potentially pain free (12). In addition to the clinical benefit of minimally invasive strategies for vaccination, the manufacturing and stability advantages of genetic constructs potentially offers a more efficient, lower cost and mass-distributable means of vaccination compared to conventional approaches (13). This is of particular relevance for third world countries requiring mass vaccination programs, western countries recently immersed in a climate of threats from bioterrorism and globally, with the recent concern of a new influenza pandemic.

Previously, devices consisting of an array of microprojections have been shown to be capable of facilitating the delivery of pDNA based vaccines to animal models (14). In addition, in our laboratories, we have recently demonstrated that microneedles can facilitate the delivery of pDNA to the epidermis of human skin where it is taken up by viable epidermal cells and subsequently expressed (8,9). In this study we aimed to investigate whether microneedles could be used

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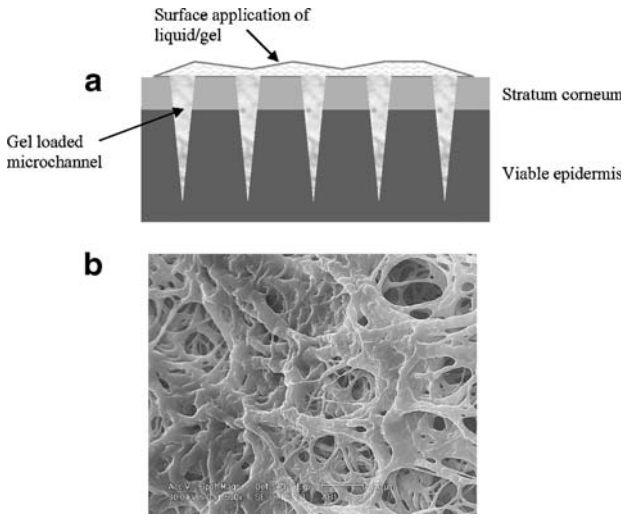

Fig. 1. **a** A schematic illustrating microneedle delivery of a pDNA loaded hydrogel to the epidermis. In the case of Carbopol the skin will be surface treated with gel, whereas for the thermosensitive gel the skin will be surface treated with a liquid which will form a gel in individual microchannels at increased temperature. **b** A dehydrated Carbopol-940 hydrogel observed by SEM.

in conjunction with a sustained release hydrogel formulation of pDNA to address the low gene expression efficiency often associated with non-viral gene delivery. In principle, the hydrogel provides a reservoir of pDNA, enabling migration and release over time, supplying fresh pDNA for cellular transfection. It was proposed that the micro-channels created by the microneedle device could be loaded with the pDNA hydrogel, which would act as a sustained release depot (Fig. 1a) (15,16). In this study, two distinct hydrogels were investigated, the first based on polyacrylic Carbopol-940 polymers and the second on a triblock copolymer of poly (lactide-co-glycolide) (PLGA) and poly (ethylene glycol) (PEG), i.e. poly (ethylene glycol-b-[DL-lactic acid-co-glycolic acid]-b-ethylene glycol) (PLGA-PEG-PLGA).

Carbopol is available commercially as a dehydrated powder. In this form the polymer consist of tightly coiled acrylic acid homopolymeric chains. Upon hydration, the polyacrylic acid polymers begin to uncoil and, once neutralized, form extended structures with a mesh-like appearance (Fig. 1b). Carbopol hydrogels have been shown to harbour and release low molecular weight drugs (17,18) as well as macromolecules including DNA (19). PLGA-PEG-PLGA triblock copolymers are particular useful materials since they can demonstrate thermosensitive behaviour. Specifically, they can be designed to undergo a liquid to gel change in physical state with increasing temperature (20,21). Thus polymer solutions that are low viscosity liquids at room temperature (~25°C) rapidly form a gel when the temperature is elevated to physiological temperatures (22). As an example, liquid formulations have been prepared that can be delivered hypodermically (20), but form a semi-solid gel reservoir following injection. A further benefit of these materials is that they are biodegradable, or bioeliminable, so that release of a therapeutic agent and subsequent elimination of the polymer can be controlled (23–25). Such polymer based hydrogels have previously been shown to be able to house and release pDNA over an extended period. Our novel approach was to produce an array of polymeric micro-reservoirs located at an appropriate level in the skin.

Microfabricated needles can be manufactured from a range of materials, including silicon (1,2), glass (26), metal (26) and polymers (27). The microneedles used in this study were manufactured from silicon wafers using the relatively simple and cost-effective method of wet-etching with potassium hydroxide. This reproducible process results in the formation of extremely uniform and robust arrays of microneedles that are ideally suited for application to human skin. Additionally, the process can be manipulated so that needles can be prepared with variant tip morphologies. Needle-tips were manufactured with different degrees of sharpness, from extremely sharp to a flat tipped "frustum" design. Previous studies showed that the frustum type needles create more discernable microchannels through the SC than sharp tipped needles (8) and therefore frustum tipped microneedles were used throughout this study.

MATERIALS AND METHODS

Materials

Amplification and purification of the pCMVβ reporter plasmid were as described previously (28). DL-Lactide (3, 6-dimethyl-1, 4-dioxane-2, 5-dione), stannous 2-ethylhexanoate, poly (ethylene glycol) (PEG) 1000, amine modified fluorescent nanoparticle suspension (L-1280), Hoechst 33258 (Bisbenzimide), deuterated chloroform ($CDCl_3$), TRI reagent® and constituents of the X-gal staining solution were from Sigma-Aldrich Chemical Company (Poole, UK). Triethanolamine (TEA) and Carbopol-940 were from Acros Organics (Geel, Belgium). Glycolide (1, 4-dioxane-2, 5-dione) was received as a gift from Purac (Gorinchem The Netherlands). All culture plastics were from Corning-Costar (High Wycombe, UK). Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin solution were from Invitrogen Corporation (Paisley, UK). Histological materials were from RA Lamb Ltd (Eastbourne, UK). DNA-free™ kit was from Ambion (Cambridgeshire, UK) and the one step RT-PCR kit from Qiagen Ltd (Crawley, UK). Other materials were of analytical grade and from Fisher Scientific UK (Loughborough, UK).

Methods

Microneedle Manufacture

Silicon microneedles were prepared using a previously reported approach (8, 29). Briefly, a silicon wafer was coated with a nitride and oxide layer using low pressure chemical vapour deposition (LPCVD). This layer was then lithographically patterned using plasma etching. The patterned wafer was subsequently etched using a 29% potassium hydroxide solution at a temperature of 79°C in a water bath with constant agitation. The aspect ratio of the resulting microneedles was 3:2 (height:base diameter). In a final step,

the microneedles were surface coated with titanium, as an adhesive layer, and then platinum by evaporation.

Microneedle Characterisation

Individual microneedle arrays were mounted onto metal stubs with double-sided carbon tape and visualised using a Philips XL-200 scanning electron microscope (SEM) (FEI Company, Eindhoven, The Netherlands).

Microneedle Treatment of Heat Separated Epidermal Membrane

A sample of human breast tissue (female donor aged 63) was obtained following surgery with full ethical committee approval and informed patient consent. The subcutaneous fat was removed and the excised skin dissected into areas of approximately 2 cm^2. The skin samples were then placed into a 60°C water bath for 55 s and the epidermal sheet (SC and viable epidermis) carefully removed using blunt forceps. The heat separated epidermal sheets were placed back onto the dermis and the microneedle array applied using a single rolling motion. Subsequently, the heat separated epidermal sheets were fixed in 2% glutaraldehyde and dehydrated in an alcohol gradient (70, 80, 90 and 100% each for 30 min). The sheets were then mounted onto a metal stub with double-sided carbon tape and sputter coated with gold prior to SEM.

Determination of Trans-epidermal Water Loss (TEWL)

A frozen (−20°C) human breast skin sample (female donor aged 56) was allowed to defrost for 2 h, blotted dry and equilibrated for a further 30 min. Trans-epidermal water loss (TEWL) measurements were performed on untreated skin, skin treated with a single application of a 26 G hypodermic needle and skin treated with a frustum tipped microneedle array using an opened chamber TEWL probe connected to a DERMA-LAB meter (Cortex Technology, Hadsund, Denmark).

Preparation of Loaded and Control Carbopol-940 Hydrogel

Stock solutions of pDNA were diluted to an appropriate concentration in a specified volume, typically 0.5 ml. This solution was added to a pre-weighed quantity of Carbopol-940 polymer, mixed with a glass rod for 2 min, and allowed to fully swell for approximately 1 h to yield a hydrogel. Typically a 1% w/v polymer solution was prepared. Fluorescent nanoparticle (100 nm) loaded Carbopol-940 hydrogels were also prepared in the absence of pDNA using the same procedure. All Carbopol-940 hydrogels were neutralised (pH 6.5–7) with triethylamine (TEA).

Synthesis of PLGA-PEG-PLGA Tri-block Co-polymers and Preparation of Hydrogels

Synthesis of the PLGA-PEG-PLGA tri-block co-polymer was performed by ring opening polymerisation of lactide and glycolide using a stannous 2-ethylhexanoate catalyst (30). Briefly, Polyethylene glycol 1000 (7.5 g) was dried in a three-neck flask under vacuum at 150°C for 3 h. DL-lactide (14.1 g) and glycolide (3.8 g) were added (3:1 molar ratio), and the flask heated at 150°C for a further 30 min. To the reaction 50 μg of stannous 2-ethylhexanoate was then added and the temperature increased to 155°C where it was maintained for 5 h. The resulting crude polymer mix was dissolved in 500 ml of ice cold water and precipitated by heating at 80°C (this step was repeated a total of three times). Subsequently, the polymer was dehydrated by freeze drying and stored in a dried state at 4°C.

Stock solutions of pDNA were diluted to an appropriate concentration in a specified volume, typically 1 ml. This solution was added to a pre-weighed quantity of PLGA-PEG-PLGA polymer and mixed. The polymer was then incubated at 4°C overnight to yield a polymer solution. Typically a 23% w/w polymer solution was used during this study. The sol-gel transition temperature of the polymer solution was determined by a tube inversion test based on a method described by Jeong *et al.* (31).

^1H Nuclear Magnetic Resonance (NMR) Analysis

Approximately 10 mg of PLGA-PEG-PLGA tri-block co-polymer was dissolved in $CDCl_3$. NMR spectra were obtained using a Bruker 300 MHz NMR spectrometer (Bruker, Coventry, UK).

Gel Permeation Chromatography (GPC) Analysis

Approximately 10 mg of PLGA-PEG-PLGA tri-block co-polymer was dissolved in 1 ml of tetrahydrofuran (THF) containing 20 μl of toluene by stirring at room temperature for approximately 30 min. Samples were analyzed at ambient temperature using an integrated PL-GPC 20 GPC system (Polymer Laboratories Ltd, Shropshire, UK) with two ResiPore columns (particle size 3 μm, both 7.5 mm×300 mm) in series (Polymer Laboratories Ltd, Shropshire, UK) with detection via a deflection refractive index detector. THF was employed as a mobile phase with a flow rate of 1 ml/min and molecular weight of the polymer was determined relative to polystyrene standards.

Microneedle Facilitated Delivery of Fluorescent Nanoparticle Loaded Hydrogels to Skin

A human breast skin sample (female donor aged 62) was removed from −20°C storage and allowed to defrost and equilibrate to room temperature for ~2 h. Approximately 20 μl of nanoparticle loaded hydrogel (Carbopol 1% w/v and PLGA-PEG-PLGA 23% w/w in 1 ml of 0.5% aqueous nanoparticle stock solution) was applied to the surface of the excised human skin. A frustum-tipped microneedle device was applied to the hydrogel treated area using a single rolling motion whereby the needles were positioned at an angle of approximately 45° to the skin surface and rotated forward through an angle of approximately 90°, finishing at a 45° angle to the skin surface in the opposing direction. Consistent downward pressure was maintained throughout the application. Following treatment, the skin samples were incubated at 37°C for 15 min before the surface was washed in PBS, for 30 min, and then fixed in 2% glutaraldehyde for 2 h at 4°C. Residual fixative was removed by washing with PBS. Samples were subsequently embedded in OCT medium and stored at −80°C.

Diffusion of pDNA from Hydrogel Systems Through Heat Separated Epidermal Sheet

1% w/v Carbopol-940 and a 23% w/w PLGA-PEG-PLGA hydrogels were prepared, each containing 1 mg/ml pCMVβ. The donor compartment of a static Franz type diffusion cell was loaded with 1 ml of hydrogel and occluded with foil. The diffusive membrane was a heat separated epidermal sheet (prepared as described previously and treated once with the microneedle array) clamped between the donor and receptor phase and sealed with silicon grease. The receptor phase of each diffusion cell was filled with ~3 ml of TNE buffer (100 mM Tris; 2.0 M NaCl; 10 mM EDTA; pH 7.4) and magnetically stirred for 72 h in a 37°C water bath. At pre-determined time intervals, 200 µl samples were removed from the receptor phase which was replenished with an equal volume of TNE buffer. Plasmid permeation was monitored by measuring the total amount of DNA in each sample using Hoeschst DNA quantification (32). Fluorescence was measured using a FLUOstar Optima fluorimeter (BMG Labtechnologies, Offenberg, Germany).

Demonstration of Organ Culture Viability Over 24 H

Immediately following surgical removal, human breast tissue from a 64 year old female patient was transferred into full culture media at 4°C and transported to the laboratory where the subcutaneous fat and the majority of the dermis were removed by careful dissection, resulting in 'split-thickness' skin. The SC was disrupted in two distinct regions, and each subsequently received 20 µg of pCMVβ. One region was immediately placed in RNA*later*® and stored at −20°C. The other sample was cultured as reported previously (8) for 24 h before being immersed in RNA*later*® and stored at −20°C. Total RNA was isolated from each sample using TRI Reagent® and contaminating genomic DNA removed by treatment with DNA-free™ kit as described previously (REF). RT-PCR reactions were performed, using 2 µg of total RNA, on both samples using primers specific for a 400 bp fragment of the β-gal transcript (5'-TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA-3' and 5'-ATG TGA GCG AGT AAC CCG TCG GAT TCT-3'). Reaction products were run on a 1% agarose gel, containing eithidium bromide, at 100 V for 1 h before observation with a UV gel doc (8).

Skin Organ Culture and pDNA Delivery

All gene delivery studies were performed on recently excised human breast skin from a 62 year old female donor. Each hydrogel formulation (100 µl containing 100 µg of pCMVβ reporter pDNA) was then applied and spread over a limited surface (~1 cm^2) of the split-thickness skin prior to application of the frustum-tipped microneedle device in a single rolling motion, i.e. the array is applied at an acute angle of 45° to the skin, rolled through an angle of 180° continuing to an obtuse angle of approximately 135° (n=4 for both types of hydrogel) on the same area. Other regions of the skin also received 100 µl of each hydrogel formulation spread over the surface but spread over an area ~1 cm×2 cm. The microneedle array was applied directly to the skin surface and dragged in a lateral scrap for ~2 cm, through the hydrogel formulation (n=2 for each hydrogel). The treated areas of the skin were dissected and cultured in full culture media (94% DMEM : 5% FBS : 1% penicillin/streptomycin) at 37°C and 5% CO_2 for 24 h using a previously validated organ culture system (8).

After 24 h, all samples were rinsed in PBS/$MgCl_2$ for 30 min, and fixed in 2% glutaraldehyde/PBS/$MgCl_2$ at 4°C for 2 h. Subsequently, each sample was rinsed three times in PBS/$MgCl_2$ for 2 h. Samples were then immersed in X-Gal staining solution (0.2% X-Gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ prepared in PBS) for 24 h at 37°C. Points of expression were counted on each sample by observation en face using a Stemi 2000-C Stereomicroscope (Zeiss, Welwyn Garden City, UK) and a KL1500 electronic external light source (Schott UK Limited, Stafford, UK), prior to each sample being embedded in OCT medium and stored at −80°C.

Preparation of Skin Cryosections and H&E Staining

Samples embedded in OCT blocks were sectioned using a Leica CM3050S Cryostat (Leica Microsystems, Milton Keynes, UK). Skin sections (10 or 12 µm) were captured onto Superfrost Plus® microscope slides, dried overnight and observed with an Olympus BX50 microscope (Olympus, Middlesex, UK). Selected slides were further subjected to haematoxylin and eosin (H&E) staining to assist visualisation of skin architecture.

RESULTS AND DISCUSSION

The wet etched microneedle arrays used in this study had a surface area of ~3 mm^2 and contained 16 microneedles equally distributed in a 4×4 arrangement. These were initially characterised by SEM (Fig. 2a). Although the wet etching process is commonly prone to poor reproducibility, the microneedle arrays used in this study were shown to be regular and reproducible through effective process control (11,27). At increased magnification, the structure of individual needles was apparent (Fig. 2b). Each needle was ~260 µm in height, with a base diameter of ~200 µm, and a flattened tip (frustrum) ~100 µm in diameter. This type of

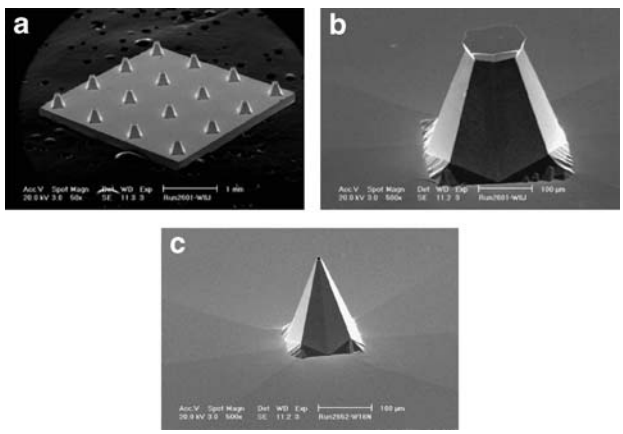

Fig. 2. SEM images of microneedle arrays at different magnifications. **a** The microneedle array used in the study (*bar* = 1 mm). **b** A frustum tipped microneedle array. **c** A sharp tipped microneedle (*bar* = 100 µm in both cases).

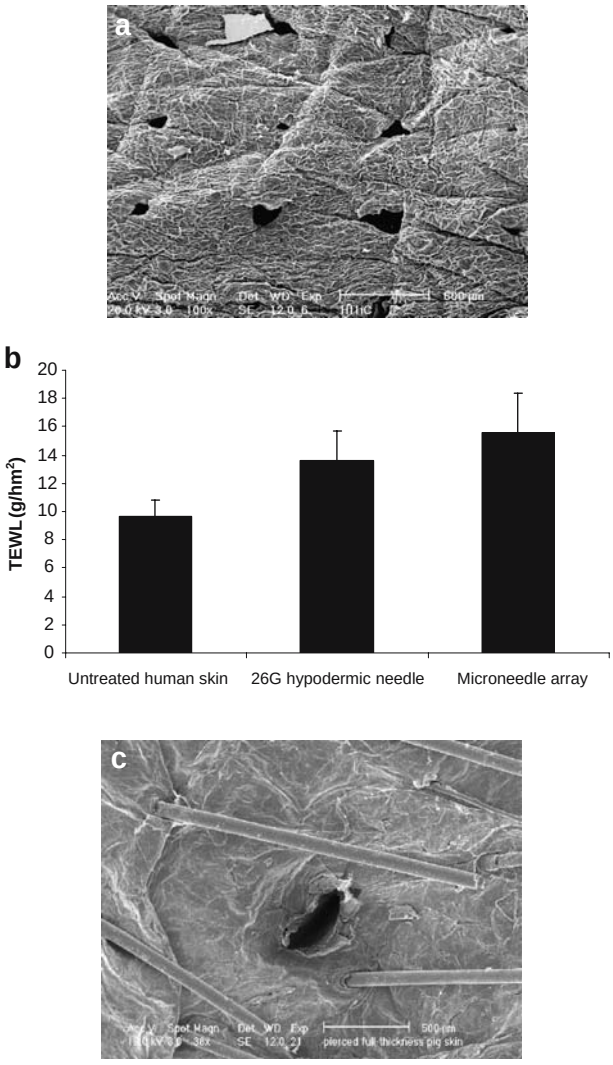

Fig. 3. **a** Disruption of the SC in a heat separated epidermal membrane resulting from microneedle application as observed by SEM (*bar* = 500 μm). **b** Loss of SC integrity in full thickness skin determined by TEWL. **c** An SEM image of porcine skin treated with a hypodermic needle (*bar* = 500 μm).

needle was used in preference to sharp tipped microneedles (Fig. 2c) as preliminary studies had indicated that this type of microneedle geometry resulted in improved gene expression due to increased transient disruption to the SC and possibly temporary compromise of keratinocyte membranes (8). Disruption of the SC, following frustum tipped microneedle treatment, was clearly visible when heat separated epidermal sheets, (SC and viable epidermis), were removed from treated skin (Fig. 3a). Membrane punctures, typically between 50–100 μm in diameter, corresponding to the spatial pattern of the microneedle array were clearly visible.

Microneedle induced disruption of full thickness skin was assessed by comparing the trans-epidermal water loss (TEWL) of treated skin with that of untreated control skin. It was predicted that microneedle treatment would result in a decrease in barrier function and a related increase in TEWL. A significant ($P<0.05$) increase in TEWL was observed for microneedle treated skin as compared to non-treated control skin (Fig. 3b). Skin samples that had received a single puncture with a 26 G hypodermic needle demonstrated statistically ($P<0.05$) comparable water loss to those samples treated with a single application of the 16-microneedle array. Interestingly, this observation directly correlates with the total area of punctures. Figure 3a shows that, although variable in dimensions, typically microneedle punctures have a diameter of 50–100 μm. Therefore, based on the assumption that one microneedle application creates 16 punctures, each of ~75 μm diameter, the total puncture area (or area of disruption) is $\sim 70\times10^3$ μm^2. When a similar calculation was carried out for the disruption to the skin surface (in this case porcine skin) caused by a 26 G hypodermic needle (Fig. 3c) (diameter of puncture ~300 μm) the total puncture area was essentially the same.

The PLGA-PEG-PLGA tri-block copolymer was initially characterised by ^{1}H-NMR spectroscopy and GPC. A typical ^{1}H-NMR spectrum is shown in Fig. 4. Spectra of peaks corresponding to the methine and methyl hydrogen of the DL lactide constituent (5.2 and 1.5 ppm, respectively), the methylene hydrogen of the glycolide component (4.8 ppm), and the methylene hydrogen of the PEG (3.6 ppm) were observed, which are consistent with the finding of Chen *et al.* (33). The ratio of DL Lactide to glycolide was determined, by ^{1}H-NMR, to be 1.74 (Table I) which suggests a relatively high glycolide component, a determinant of resultant release properties.

The molecular weight and polydispersity index of the PLGA-PEG-PLGA tri-block copolymer was determined by GPC as 3882 and 1.14, respectively (Table I). Polymers within this range have been shown to display thermo-reversible sol-gel transitions when present in specific concentrations in aqueous solutions (33). Below the lower critical solution temperature (LCST) the gel reversibly returns to a fluid state and behaves as a Newtonian fluid (30), but when heated forms a gel. In this instance the gelation point was found to be ~32°C for a 23% w/w solution as determined by test tube inversion (data not shown). At temperatures above 40°C, the polymer precipitated, but when cooled below the

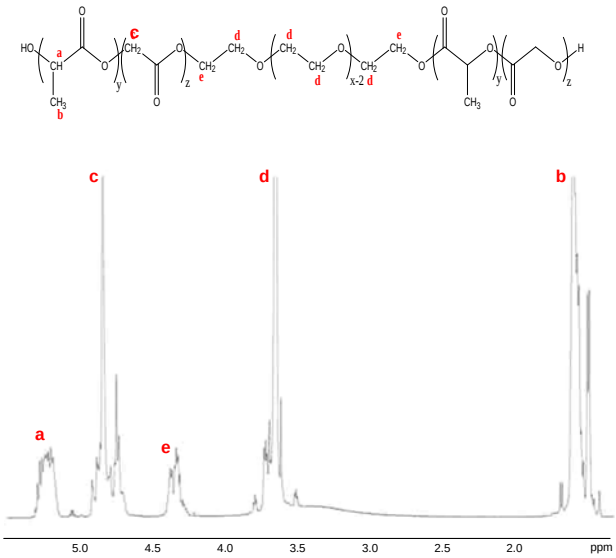

Fig. 4. A typical ^{1}H NMR spectra for PLGA-PEG-PLGA triblock copolymer.

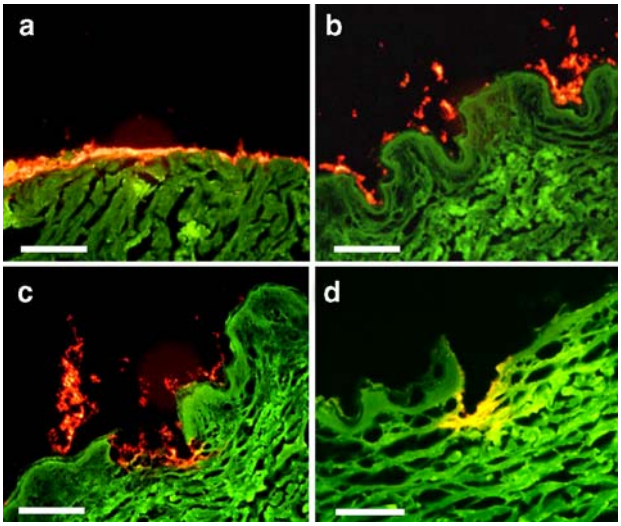

Fig. 5. Cryosections of *ex vivo* human skin observed by fluorescent microscopy. **a**, **b** Application of a 1% *w/v* Carbopol hydrogel containing red fluorescent nanobeads to intact skin. Microneedle application removes the SC integrity and the hydrogel formulations (**c** and **d**) are able to reach the epidermis: **c** a 1% *w/v* Carbopol-940; **d** a 23% *w/w* PLGA-PEG-PLGA hydrogel (*bar* = 100 μm in all cases).

LCST reverted back to a solution which was able to reform a gel on heating.

We have previously used 100 nm fluorescent latex nanoparticles as a readily identifiable model for non-viral gene delivery vectors, for example LPD (2). However, in this study these fluorescent latex nanoparticles were used as a marker within the hydrogel which demonstrated that the hydrogel resided within the formed microconduits. Figure 5 shows 10 μm cryosections of ex vivo human skin treated with hydrogels loaded with fluorescent nanoparticles observed by fluorescent microscopy. The green fluorescence observed in all sections was a consequence of autofluorescence of skin components and not the result of staining. Both types of hydrogels failed to penetrate the SC in regions where the microneedles were not applied, with the red fluorescent signal emitted from the nanoparticle loaded hydrogel restricted to the external surface of the SC (Fig. 5a,b). In some cases, the gel could be observed residing in skin ridges and other natural folds although the SC was still seen to be intact and no underlying red fluorescence was observed (Fig. 5b). However, when the hydrogel/nanoparticle formulations were applied to skin in conjunction with microneedles, nanoparticles were observed within the viable epidermis. Red fluorescence due to the nanoparticles was observed to be localised to microneedle created channels. This demonstrated that the application of the microneedles to the surface of the skin had penetrated the skin surface and pushed the nanoparticle containing hydrogel formulations into the resulting microchannels. (Fig. 5c,d). Significant disruption to the SC was clearly evident in these images, leading to the formation of conduits with typical depths of 150–200 μm and nanoparticles could clearly be observed as red or yellow (combination of red nanoparticle and green autofluorescence) against the green autofluorescent background within the viable epidermis.

The temporal release of pDNA from the hydrogel delivery systems was determined using Franz-type diffusion cells (Fig. 6). Hydrogels (23% w/w PLGA-PEG-PLGA; 1% Carbopol-940) containing pDNA 1 mg/ml were loaded into the donor compartment of diffusion cells with heat separated epidermal sheets, treated with a single application of the microneedle array, as the diffusive membrane. Release of pDNA from the hydrogel and the resulting diffusion into the receptor phase was determined over 5 days at 37°C. The migration of a control formulation of aqueous pDNA across the microneedle treated epidermal sheet was generally observed to be lower than the hypothetically expected values. This suggested that the channels were either becoming obstructed by debris released from the epidermal sheet or possibly that the channels were closing, due to hydration of the epidermal sheet which resulted in swelling and subsequent constriction of the channels. Nevertheless, the diffusion of pDNA was retarded when incorporated into both types of hydrogel. Release and subsequent diffusion was significantly greater from the PLGA-PEG-PLGA based hydrogel; with release and diffusion from the Carbopol hydrogel being negligible in comparison over this time period using this detection system. The mechanism of pDNA release would be expected to be different for the two types of hydrogel. In both hydrogels, migration of pDNA through the gel matrix would determine release rate. However, in the thermosensitive hydrogel, the PLGA-PEG-PLGA polymers are prone to hydrolysis of their ester bonds so that the integrity of the polymer matrix is lost relatively quickly (compared to Carbopol) at physiological temperatures. Therefore, for the PLGA-PEG-PLGA hydrogel matrix, both migration and erosion effects will determine pDNA release and subsequent diffusion (34). At present we are unable to predict the relative contribution of each of these effects.

Demonstration of proof-of-principle that hydrogel formulations containing pDNA can be delivered to the viable epidermis following application of microneedles was achieved using ex vivo skin explants. The ex vivo organ culture system used in these studies has been described previously (8). Confirmation that the organ culture system

Table I. Molecular Weight and PL/GA Ratio of the Synthesised PLGA-PEG-PLGA Triblock Copolymer as Determined by GPC and ^{1}H-NMR

Molecular Weight of PLGA-PEG-PLGA Polymers		DL-Lactide/Glycolide Ratio[a]	Polydispersity Index[b]
M_w^a	M_n^a		
3,882	2,986	1.24	1.139

[a] Determined by ^{1}H-NMR
[b] Determined by GPC

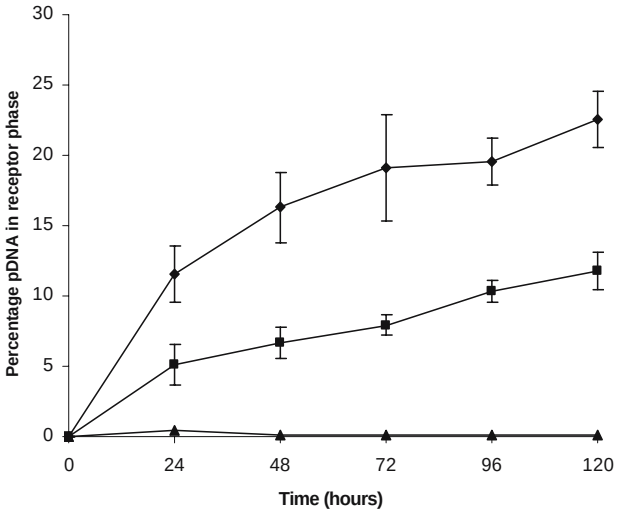

Fig. 6. The migration of pDNA through a microneedle treated human heat separated epidermal sheet: a solution of pDNA (*filled diamond*), a 1% *w/v* Carbopol hydrogel (*filled triangle*) and a 23% *w/w* PLGA-PEG-PLGA triblock copolymer hydrogel (*filled square*).

retains skin viability over the incubation period was demonstrated using RT-PCR specific for a 400 bp fragment of the β-galactosidase transcript. Figure 7 shows RT-PCR products of skin samples treated with pCMVβ and processed either immediately after treatment and following 24 h incubation in organ culture. The results show that the skin is still genetically viable at 24 h. Beyond this timepoint decreasing cellular viability becomes an issue, therefore to date all gene delivery studies performed in ex vivo human skin are performed over 24 h. Figure 8a,b shows en face images of *ex vivo* human skin treated with pCMVβ loaded hydrogel formulations, Carbopol-940 (a) and PLGA-PEG-PLGA (b), and wet-etch silicon microneedles with subsequent immersion in X-Gal staining solution. Figure 8c shows the total number of microchannels positive for gene expression following a single treatment with the hydrogels or a solution of pDNA. Both the 1% w/v Carbopol-940 and 23% w/w PLGA-PEG-PLGA tri-block copolymer hydrogels were shown to release functional pDNA which was able to transfect skin cells and express the gene product; β-galactosidase converting a substrate to reveal blue colouration. In control experiments, where pDNA hydrogels were applied to skin which had not been treated with microneedles, expression was not observed (data not shown).

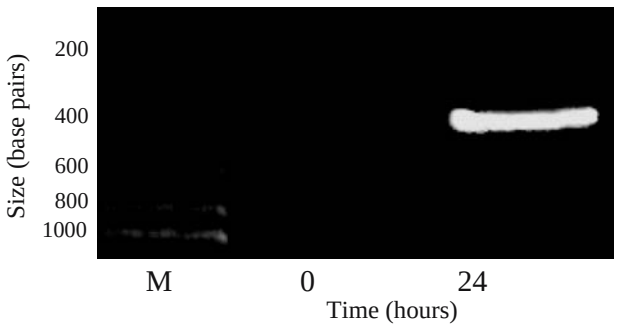

Fig. 7. RT-PCR analysis of β-galactosidase expression in *ex vivo* human skin. When RNA was isolated from samples immediately after the plasmid application (time 0) then no expression is observed. However, following culturing for 24 h, a clear signal indicating expression form the plasmid was observed, confirming that the skin remains genetically viable during the culturing period.

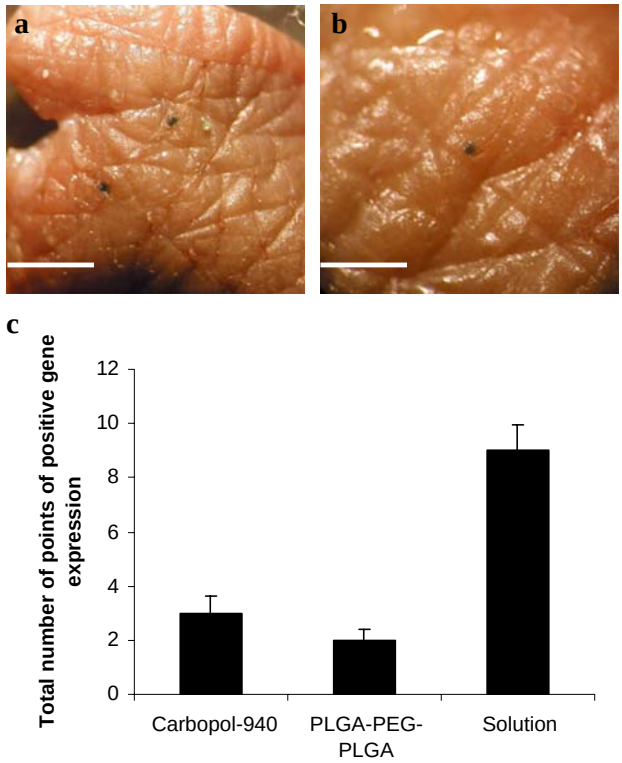

Fig. 8. Positive expression from microneedle assisted delivery of pCMVβ to *ex vivo* human skin (microneedles applied in a single rolling motion). *En face* image of skin treated with a 1% Carbopol-940 hydrogel (**a**) and 23% *w/w* PLGA-PEG-PLGA triblock copolymer hydrogel (**b**) each containing pDNA with subsequent application of microneedles (*bar* = 1 mm in both cases). **c** The total number of points of positive expression observed in samples treated with pDNA loaded hydrogels contrasted with pDNA delivered in solution.

It was noted that overall levels of gene expression were low and not significantly different between the hydrogel formulations (Fig. 8c) despite the aforementioned increase in pDNA release from PLGA-PEG-PLGA hydrogels (Fig. 6). It is possible that during hydrolysis of the PLGA-PEG-PLGA polymer hydrogel the constituent breakdown products could be reducing uptake and/or expression of pDNA either by direct physical hindrance or by chemically modifying the extracellular or intracellular environment, for example altering pH. Also, both hydrogel formulations will be gradually delivering pDNA over time and the human organ culture system that is currently employed only permits gene expression experiments to be performed for 24 h, due to restrictions of cellular viability. Clearly this may lead to an underestimation of gene expression from sustained release matrices and is currently being addressed using alternative biological models.

To determine the primary location of β-galactosidase expression in skin layers 12 μm cryosections were taken from the same samples and observed using light microscopy revealed that reporter gene expression following application of pDNA loaded hydrogels and microneedles were proximal

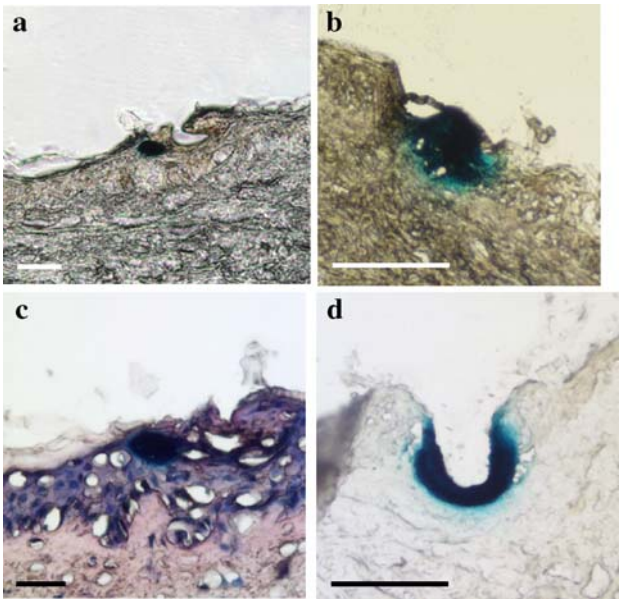

Fig. 9. Cryosections (12 μm) prepared from skin samples treated initially with either 1% Carbopol-940 (**a**, **c**) or 23% *w/w* PLGA-PEG-PLGA triblock copolymer hydrogel (**b**, **d**) and subsequently with wet-etch microneedles (*bar* = 150 μm in all cases).

to a microconduit (typically between150–200 μmin depth) and limited to cells of the epidermis, Fig. 9. The image shown in Fig. 9d, not stained with H&E, shows a micro-channel created using the frustum-tipped microneedles and loaded with thermosensitive gel. It is interesting to note that both the structural integrity of this individual microchannel, possibly a result of the loaded gel restricting tissue regress, and intensity of gene expression are more pronounced than that observed with the Carbopol gel (Fig. 9a,c) and aqueous formulations (11). Gene expression was also observed when the hydrogel formulations were applied to skin prior to lateral application of microneedles (Fig. 10a–d). The points of expression were exclusively associated with the barrier disruption caused by microneedle application (Fig. 10, arrows). While more points of expression were observed following this method of application, presumably as a result of enhanced disruption of SC and increased contact of pDNA with epidermal cells, there was no notable variance between the levels of gene expression arising from the hydrogel formulations Fig. 10e.

To date, the gene expression efficiency we have observed in microneedle treated skin has been restricted to a small number of microchannels. Typically, when a solution of pDNA was pre-applied to the surface of microneedle treated skin up to 30% of the microchannels demonstrated positive reporter gene expression (8). When the pDNA was formulated in a hydrogel this value was reduced to approximately 12%. We have previously speculated as to the reasons why gene expression efficiency in microneedle-treated human skin is relatively low. These include limited entry of the pDNA into the microchannel, inefficient cellular uptake of the pDNA, cell damage caused by the infiltrating microneedles and unreliable detection of reporter gene product due to restricted access of the staining solution to the transfected cells (8). By using a gel formulation we may be further restricting the access of pDNA to cellular targets over the initial 24 hr incubation period (as suggested in Fig. 6). As a corollary, whilst pDNA hydrogels provide potential for sustained release, such formulations may indeed *initially* result in lower levels of gene expression than would be observed with liquid systems. Whilst our organ culture system provides a valuable method for testing gene expres-

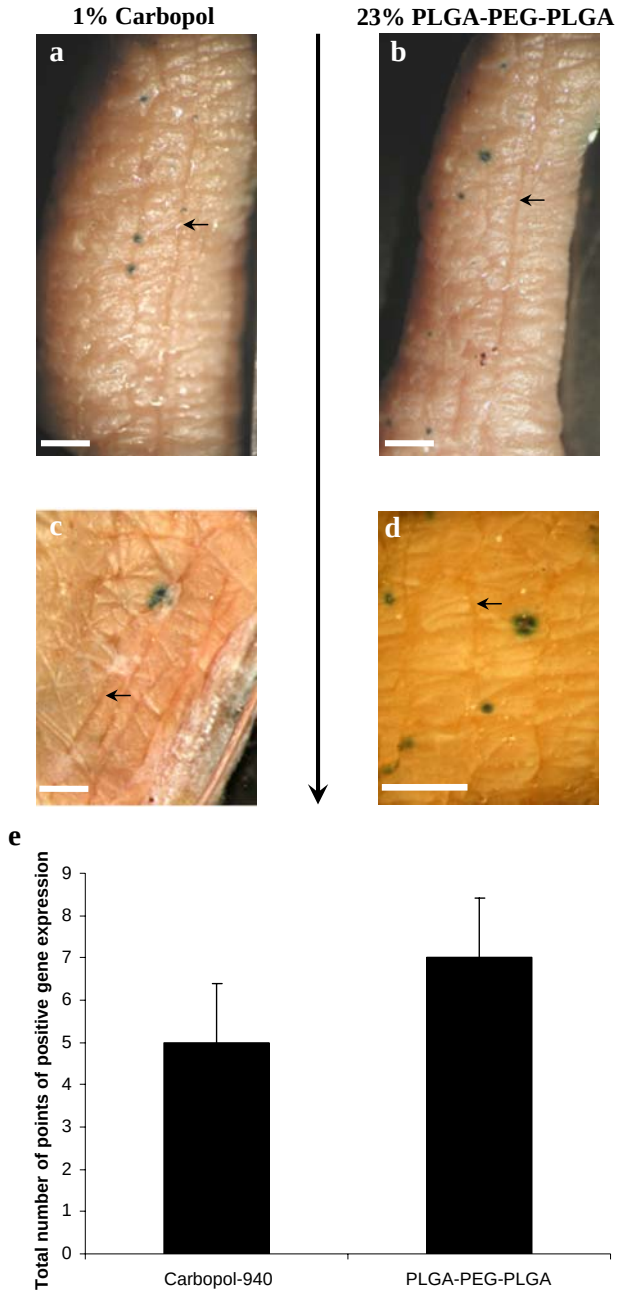

Fig. 10. Delivery of pDNA loaded hydrogels with microneedles applied in a single lateral scrape. The lines of disruption caused by the microneedles are indicated by *arrows*. Expression from pDNA loaded in 1% Carbopol-940 polymers (**a**, **c**) and 23% *w/w* PLGA-PEG-PLGA hydrogel (**b**, **d**). The *large black arrow* indicates the direction of microneedle lateral application; *bar* = 1 mm in all cases. **e** The total number of points of positive expression observed when microneedles were applied in a lateral motion ($n = 2$ in each instance).

sion in the biological and architectural human skin environment, it is currently impractical to guarantee excised human skin viability to determine gene expression over longer time periods. Further *in vivo* studies with these formulations will investigate the ability of these systems to control pDNA release for sustained gene expression.

CONCLUSIONS

In this study we have shown the ability to create channels through the SC using microfabricated needles and mediate reporter gene expression in viable human skin using pDNA loaded hydrogels. The frustum type microneedle array exploited in these studies was proficient in creating channels of sufficient dimensions for the passage of relatively large materials, such as macromolecular nanoparticles and pDNA, whilst being of sufficient robustness for repeated use. The hydrogel delivery systems were able to harbour pDNA in the microneedle-facilitated microchannel and release the plasmid in a functional form for gene expression in the viable epidermis. The combination of the microneedle physical delivery strategy and sustained release formulation could potentially be employed for clinical applications where it is an advantage for large molecules to enter the viable epidermis over prolonged periods, for instance in genetic vaccination.

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REFERENCES

1. S. Henry, D. V. McAllister, M. G. Allen, and M. R. Prausnitz. Microfabricated microneedles: a novel approach to transdermal drug delivery. *J. Pharm. Sci.* **87**:922–925 (1998).
2. F. Chabri, K. Bouris, T. Jones, D. Barrow, A. Hann, C. Allender, K. Brain, and J. Birchall. Microfabricated silicon microneedles for nonviral cutaneous gene delivery. *Br. J. Dermatol.* **150**:869–877 (2004).
3. W. Martanto, S. P. Davis, N. R. Holiday, J. Wang, H. S. Gill, and M. R. Prausnitz. Transdermal delivery of insulin using microneedles *in vivo*. *Pharm. Res.* **21**:947–952 (2004).
4. J. A. Matriano, M. Cormier, J. Johnson, W. A. Young, M. Buttery, K. Nyam, and P. E. Daddona. Macroflux® microprojection array patch technology: a new and efficient approach for intracutaneous immunization. *Pharm. Res.* **19**:63–70 (2002).
5. R. K. Sivamani, B. Stoeber, G. C. Wu, H. Zhai, D. Liepmann, and H. Maibach. Clinical microneedle injection of methyl nicotinate: stratum corneum penetration. *Skin Res. Technol.* **11**:152–156 (2005).
6. J. H. Park, M. G. Allen, and M. R. Prausnitz. Polymer microneedles for controlled-release drug delivery. *Pharm. Res.* **23**:1008–1019 (2006).
7. H. S. Gill and M. R. Prausnitz. Coated microneedles for transdermal delivery. *J. Control. Release* **117**:227–237 (2007).
8. J. C. Birchall, S. A. Coulman, M. Pearton, C. Allender, K. Brain, A. Anstey, C. Gateley, N. Wilke, and A. Morrissey. Cutaneous DNA delivery and gene expression in ex vivo human skin explants via wet-etch microfabricated microneedles. *J. Drug Target.* **13**:415–421 (2005).
9. S. A. Coulman, D. Barrow, A. Anstey, C. Gateley, A. Morrissey, N. Wilke, C. Allender, K. Brain, and J. C. Birchall. Minimally invasive cutaneous delivery of macromolecules and plasmid DNA via microneedles. *Curr. Drug Discov.* **3**:65–75 (2006).
10. I. R. Williams and T. S. Kupper. Immunity at the surface: homeostatic mechanisims of the skin immune system. *Life Sci.* **58**:1485–1507 (1996).
11. E. F. Fynan, R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. DNA vaccines: protective immunizations by parenteral, mucosal and gene-gun innoculations. *Proc. Natl. Acad. Sci. U. S. A.* **90**:11478–11482 (1993).
12. S. Kaushik, A. H. Hord, D. D. Denson, D. V. McAllister, S. Smitra, M. G. Allen, and M. R. Prausnitz. Lack of pain associated with microfabricated microneedles. *Anesth. Analg.* **92**:502–504 (2001).
13. M. T. S. Lin, L. Pulkkinen, and J. Uitto. Cutaneous gene therapy—principles and prospects. *Dermatol. Clin.* **18**:177–187 (2000).
14. J. A. Mikszta, J. B. Alarcon, J. M. Brittingham, D. E. Sutter, R. J. Pettis, and N. G. Harvey. Improved genetic immunization via micromechanical disruption of skin-barrier function and targetted epidermal delivery. *Nat. Med.* **8**:415–419 (2002).
15. D. W. Pack, A. S. Hoffman, S. Pun, and P. S. Stayton. Design and development of polymers for gene delivery. *Nat. Rev. Drug Discov.* **4**:581–593 (2005).
16. L. D. Shea, E. Smiley, J. Bonadio, and D. J. Mooney. DNA delivery from polymer matrices for tissue engineering. *Nat. Biotechnol.* **17**:551–554 (1999).
17. I. Csoka, E. Csanyi, G. Zapantis, E. Nagy, A. Feher-Kiss, G. Horvath, G. Blazso, and I. Eros. *In vitro* and *in vivo* percutaneous absorption of topical dosage forms: case studies. *Int. J. Pharm.* **291**:11–19 (2005).
18. P. Mura, G. P. Bettinetti, A. Liguori, and G. Bramanti. Improvement of clonazepam release from a Carbopol hydrogel. *Pharm. Acta Helv.* **67**:282–288 (1992).
19. F. A. Ismail, J. Napaporn, J. A. Hughes, and G. A. Brazeau. *In situ* gel formulations for gene delivery: release and myotoxicity studies. *Pharm. Dev. Technol.* **5**:391–397 (2000).
20. B. Jeong, Y. H. Bae, D. S. Lee, and S. W. Kim. Biodegradable block copolymers as injectable drug-delivery systems. *Nature* **388**:860–862 (1997).
21. M. S. Shim, H. T. Lee, W. S. Shim, I. Park, H. Lee, T. Chang, S. W. Kim, and D. S. Lee. Poly(D,L-lactic acid-co-glycolic acid)-b-poly(ethylene glycol)-b-poly (D,L-lactic acid-co-glycolic acid) triblock copolymer and thermoreversible phase transition in water. *J. Biomed. Materi. Res.* **61**:188–196 (2002).
22. P. Lee, Z. Li, and L. Huang. Thermosensitive hydrogel as a Tgf-β1 gene delivery vehicle enhances diabetic wound healing. *Pharm. Res.* **20**:1995–2000 (2003).
23. J. Nieuwenhuis. Synthesis of polylactides and their copolymers. *Clin. Mater.* **10**:59–67 (1992).
24. B. Jeong, Y. H. Bae, and S. W. Kim. *In situ* gelation of PEG-PLGA-PEG triblock copolymer solutions and degredation thereof. *J. Biomed. Materi. Res.* **50**:171–177 (2000).
25. W. Amass, A. Amass, and B. Tighe. A review of biodegradable polymers: uses, current developments in the synthesis and characterization of biodegradable polyesters, blends of biodegradable polymers and recent advances in biodegradation studies. *Polym. Int.* **47**:89–144 (1998).
26. D. V. McAllister, P. M. Wang, S. P. Davis, J. H. Park, P. J. Canatella, M. G. Allen, and M. R. Prausnitz. Microfabricated needles for transdermal delivery of macromolecules and nanoparticles: fabrication methods and transport studies. *Proc. Natl. Acad. Sci. U. S. A.* **100**:13755–13760 (2003).
27. J. H. Park, M. G. Allen, and M. R. Prausnitz. Biodegradable polymer microneedles: fabrication, mechanics and transdermal drug delivery. *J. Control. Release.* **104**:51–66 (2005).
28. J. C. Birchall, I. W. Kellaway, and M. Gumbleton. Physical stability and *in-vitro* gene expression efficiency of nebulised lipid-peptide-DNA complexes. *Int. J. Pharm.* **197**:221–231 (2000).
29. N. Wilke, A. Mulcahy, S.-R. Ye, and A. Morrissey. Process optimisation and characterisation of silicon microneedles fabri-

cated by wet etch technology. *Microelectron. J.* **36**:650–656 (2005).
30. G. M. Zentner, R. Rathi, J. Shih, J. C. McRea, M. Seo, H. Oh, B. G. Rhee, J. Mestecky, Z. Moldoveanu, M. Morgan, and S. Weitman. Biodegradable block copolymers for delivery of proteins and water-insoluble drugs. *J. Control. Release* **72**:203–215 (2001).
31. B. Jeong, Y. H. Bae, and S. W. Kim. Biodegradable Thermosensitive Micelles of PEG-PLGA-PEG Triblock Copolymers. *Colloids Surf., B Biointerfaces.* **16**:185–193 (1999).
32. K. Rengarajan, S. M. Cristol, M. Mehta, and J. M. Nickerson. Quantifying DNA concentrations using fluorometry: a comparision of fluorophores. *Mol. Vis.* **8**:416–421 (2002).
33. S. Chen, R. Pieper, D. C. Webster, and J. Singh. Triblock copolymers: synthesis, characterization, and delivery of a model protein. *Int. J. Pharm.* **288**:207–218 (2005).
34. Z. Li, W. Ning, J. Wang, A. Choi, P. Y. Lee, P. Tyagi, and L. Huang. Controlled gene delivery system based on thermosensitive biodegradable hydrogel. *Pharm. Res.* **20**:884–888 (2003).