Towards pain-free diagnosis of skin diseases through multiplexed microneedles: biomarker extraction and detection using a highly sensitive blotting method

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Towards pain-free diagnosis of skin diseases through multiplexed microneedles: biomarker extraction and detection using a highly sensitive blotting method

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DOI: 10.1007/s13346-015-0231-5

Abstract

Immunodiagnostic microneedles provide a novel way to extract protein biomarkers from the skin in a minimally invasive manner for analysis in vitro. The technology could overcome challenges in biomarker analysis specifically in solid tissue, which currently often involves invasive biopsies. This study describes the development of a multiplex immunodiagnostic device incorporating mechanisms to detect multiple antigens simultaneously, as well as internal assay controls for result validation. A novel detection method is also proposed. It enables signal detection specifically at microneedle tips and therefore may aid the construction of depth profiles of skin biomarkers. The detection method can be coupled with computerised densitometry for signal quantitation. The antigen specificity, sensitivity and functional stability of the device were assessed against a number of model biomarkers. Detection and analysis of endogenous antigens (interleukins 1α and 6) from the skin using the device was demonstrated. The results were verified using conventional enzyme-linked immunosorbent assays. The detection limit of the microneedle device, at $\leq$10 pg/mL, was at least comparable to conventional plate-based solid-phase enzyme immunoassays.

Keywords: immunodiagnostic microneedles, microprojection arrays, biomarkers, immobilised antibody, densitometry, skin diagnosis

Introduction

Biomarker-based immunoassays are specific and quantitative, and can therefore provide a powerful tool for clinical diagnosis. However, whilst immunoassays utilising liquid tissue samples (e.g. blood and urine) have well-established applications in clinical diagnostics, their widespread use in solid tissue (e.g. skin) diagnostics remains a challenge. This is because analysis depends heavily on efficient biomarker extraction from the body, which is conventionally achieved by collecting biofluid in which the biomarkers are dissolved. For solid tissues, biomarker extraction is constrained by the limited volume of biofluid that can be extracted for in vitro analysis. Consequently, solid tissue biopsies are typically excised and analysed using semi-quantitative approaches such as immunohistochemistry

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[1, 2, 3], or biomarkers are recovered ex vivo by homogenisation or solubilisation [4, 5]. Biopsy taking is an invasive procedure—it not only causes pain and distress to the patient, but also inflicts tissue trauma and entails delicate wound care. Furthermore, biopsy samples require extensive histological processing, delaying analysis.

Microneedles are solid or hollow microstructures, typically $< 1\,\text{mm}$ long, that were initially developed for drug delivery into/across the skin (reviewed in [6, 7]). They are a minimally invasive technology with the potential to replace conventional hypodermic needles, since these micron-sized needles allow superficial skin penetration, therefore resulting in minimal discomfort [8, 9]. Kendall and co-workers [10, 11, 12, 13, 14, 15] recently demonstrated efficient biomarker extraction from the skin using solid immunodiagnostic microneedle ('microprojection') arrays coated with specific capture antibodies. Whilst the authors focused on extracting circulating biomarkers, local skin biomarkers may be extracted in the same manner to aid the diagnosis of skin conditions. These devices can be considered as a solid-phase immunoassay platform miniaturised on the surface of solid microneedles. When the microneedles are inserted into the skin, the immobilised capture antibodies bind biomarkers of interest. Biomarker extraction is achieved simply by removing the microneedle devices from the skin. Unlike extracting biofluids using hollow microneedles [16, 17, 18] or swellable hydrogel microneedles that imbibe biofluids [19, 20], this approach does not extract any perceivable amount of biofluid from the skin, but isolates the biomarkers from the biofluid in situ. It therefore enables biomarker analysis from the skin without a biopsy, and can provide a valuable point-of-care diagnostic tool for various skin conditions, such as dermatitis and skin cancer.

We have developed a multiplex microneedle device coupled with a novel blotting method for biomarker analysis. By careful selection of the raw material and surface functionalisation protocol, microneedles capable of recognising specific antigens following skin insertion were prepared in monoplex and multiplex formats. Multiplexed devices offer potential benefits for more efficient diagnoses than previously reported single-antigen capture systems [10, 11, 13, 14, 15]. Using this technique, a panel of cytokines were extracted from the skin and analysed. We used computerised densitometry to quantify signals from the blots, and validated the results by conventional plate enzyme-linked immunosorbent assay (ELISA). This simple and inexpensive blotting technique enables biomarker detection with high sensitivity, specifically at the microneedle tip, which may prove useful for determining the depth profile of skin biomarkers in a rapid and minimally invasive manner. We further examined the functional stability of the microneedle devices following 30-day desiccated storage, with or without refrigeration.

Methods

Materials

All skin samples were full thickness, and were excised from the dorsal region of mice culled for unrelated work. Hairs were removed from C57BL/6 mouse skin using an electric trimmer. Alternatively, hairless CD-1 NUDE mouse skin was purchased from Charles River (Margate, UK). Sylgard® 184 silicone elastomer was from Dow Corning (Midland, MI, USA). Polylactic acid (PLA) was from Purac Biochem (Gorinchem, The Netherlands). Unless otherwise stated, antibodies, recombinant proteins and avidin-conjugated horseradish peroxidase (HRP) were from eBioscience (San Diego, CA, USA). Nunc MaxiSorp™ 96-well ELISA plates were purchased from VWR (Lutterworth, UK). All other reagents were from Sigma-Aldrich (Gillingham, UK).

Fabrication and characterisation of microneedle devices

PLA microneedle devices were produced by micromoulding [21]. Briefly, a micromould was produced from the Sylgard® 184 elastomer against the template microneedle array. PLA was then melted at 180 °C under vacuum into the micromould. Upon cooling, solid PLA microneedle devices were manually ejected from the micromould. The microneedles were then chemically surface-activated using a method described elsewhere [22, 23]. Briefly, PLA microneedles were immersed in 6 % w/v hexamethylenediamine (HMDA) for 90 seconds, followed by thorough washing with deionised water and immersion in 2 % *w/w* glutaraldehyde for 2–3 hours. At various stages of manufacture, the appearance of the microneedle devices was characterised under light microscopy and scanning electron microscopy (SEM; FEI Quanta 600 FEG). To confirm their ability to puncture the skin, the microneedles were pressed against hairless mouse skin. Puncture marks on the skin were visualised by methylene blue staining.

Antibody immobilisation

Generally, monoplex devices (those coated with the same capture antibody across the array) were incubated overnight, at 4 °C, with the desired antibody solution (5 μg/mL). For multiplex devices (where microneedles on the same array were coated with different capture antibodies), each microneedle was individually dipped into the desired antibody solution (0.5 mg/mL) 10 times. In either case, following thorough rinsing with wash buffer (phosphate-buffered saline, pH 7.4, 0.05 % Tween® 20), the microneedles were incubated with 5% w/v bovine serum albumin (BSA) to block non-specific binding sites, rinsed with wash buffer, and air-dried.

To ascertain the effect of PLA surface activation on antibody immobilisation, a model antibody (mouse IgG, Sigma-Aldrich #I5381) was immobilised on to a monoplex device as above. The device was washed thoroughly with wash buffer and incubated with a HRP-conjugated anti-mouse antibody (Sigma-Aldrich #9044). Following thorough rinsing with wash buffer, immobilisation efficiency was determined by UV/Vis spectrophotometry as described for antigen detection below.

General method for antigen capture

Antigen capture was investigated in homogenous solutions of the target antigens, as well as in excised mouse skin. Antigen solutions (100 ng/mL, unless specified otherwise) were prepared from recombinant proteins in 1 % w/v BSA solution. The microneedles were then immersed in the antigen solution, at room temperature, for 1 hour. The capture of endogenous antigens from mouse skin was performed by administering the device on to C57BL/6 mouse skin and leaving the microneedles embedded in situ for 1 hour. An intermediate model was also used, whereby a solution containing 1 μg/mL human tumour necrosis factor (TNF)-α was injected intradermally into CD1-NUDE mouse skin using a 26G hypodermic needle. Antigen capture in this model was performed as per endogenous antigen above. General method for antigen detection Following antigen capture, microneedles were incubated with the appropriate biotinylated detection antibody solution (2 μg/mL) for 2 hours, followed by avidin-conjugated HRP (2.5 μg/mL) for 1 hour. Additionally, prior to incubation with the detection antibody, devices administered on to skin samples were immersed in 3 % *w/w* H_2O_2 to inactivate any endogenous skin peroxidase that may have adhered to the microneedles. Each step described above was preceded and followed by thorough washes of the microneedle device with wash buffer. Signal quantitation was then carried out by UV/Vis spectrophotometry or a novel blotting method (described below), with or without densitometry.

For UV/Vis spectrophotometry, microneedles were removed from the base plate and immersed in a solution of o-phenylenediamine (OPD, a chromogenic substrate of HRP) for 30–40 min. Successful

antibody immobilisation was indicated by a colour change in the colourless OPD solution to yellow. All OPD solutions were prepared at 0.4 mg/mL in 50 mM phosphate citrate buffer (pH 5.0), with 0.03 % w/v $NaBO_3$. The absorbance of the OPD solution was measured at 450 nm on an Epoch microplate spectrophotometer (BioTeK, Potton, UK).

Densitometric analysis was performed in conjunction with the blotting technique detailed below. Microneedles were air-dried and placed on a paper wetted with OPD solution for 15–30 min. This allowed a yellow spot to develop at points of contact between the paper and microneedles that had successfully captured the target antigen, producing a blot pattern on the paper that corresponded to the positions of the microneedles. The blots were converted to digital images using a flatbed scanner. Densitometric analysis was performed on the images using the ImageJ software (National Institutes of Health, USA). Signal intensity was determined from the density plots as the area under the curve. Surface plots were generated using the interactive 3D surface plot plugin for signal visualisation.

Assessment of antigen specificity

Antigen capture by monoplex devices was investigated using antigen solutions containing 0.8 pg/mL–100 ng/mL of mouse interleukin (IL)-6. Following incubation with the biotinylated detection antibody and avidin-HRP, 25 microneedles were removed from each array and pooled for spectrophotometric analysis as detailed above. A signal-versus-concentration profile was plotted, from which antigen specificity and sensitivity were inferred.

Additionally, monoplex devices were either immersed in a solution containing the target antigen (human TNF-α), or applied to mouse skin into which the target antigen (1 µg/mL) had been injected intradermally. In parallel, control samples in which the capture antibody or detection antibody had been replaced with an isotype or the target antigen had been omitted from the test solution, were also included. Signals were detected using the blotting technique as detailed above. Antigen specificity was determined from the blot patterns.

For multiplex devices, antigen specificity was determined using duplex devices capable of simultaneously detecting human TNF-α and mouse IL-6 as model antigens. The devices were incubated with solutions containing either/neither/both target antigens. Signals were detected using the blotting technique. Antigen specificity was determined from the blot patterns.

Assessment of device sensitivity

Monoplex devices were incubated with solutions containing 50 pg/mL–500 ng/mL of the target antigen (human TNF-α). Likewise, multiplex devices were incubated with solutions containing 10 pg/mL–100 ng/mL of the target antigen (mouse IL-1α). The multiplex devices also incorporated positive control (biotinylated antibody, eBioscience #13-7349) and negative control (anti-human TNF-α antibody, eBioscience #14-7348) microneedles. Signals were detected using the blotting technique. Device sensitivity was expressed as the lowest antigen concentration that produced a signal.

Assessment of functional stability

Monoplex devices for mouse IL-6 were stored for 30 days in air-tight vials filled with silica gel, either at room temperature or at 4 °C. The devices were then allowed to capture recombinant mouse IL-6 from a homogenous solution, followed by antigen detection using UV/Vis spectrophotometry. As baseline controls, the same capture antibody was freshly immobilised on to microneedles that had been surface-activated on the same occasion as the test devices. The 30-day stability of immobilised

antibodies was expressed as the percentage absorbance produced by the stored microneedles, relative to the baseline controls.

Multiplex detection of endogenous skin antigens

Microneedle devices coated with capture antibodies for mouse IL-6 and IL-1α, as well as positive controls (eBioscience #13-7349) and negative controls (eBioscience #14-7348), were administered on excised C57BL/6 mouse skin and left *in situ* for 1 hour. Captured antigens were detected using the blotting method in conjunction with densitometric analysis. Antigen content in the skin samples was verified using plate ELISA.

Plate ELISA

Mouse skin samples (1 cm^2 each) from C57BL/6 mice were placed in 2 mL of PBS and homogenised using a tissue homogeniser. The homogenates were centrifuged at $1000 \times g$ for 5 min to pellet tissue debris whilst the supernatants were collected for cytokine quantitation by plate ELISA. Nunc MaxiSorp™ 96-well ELISA plates were coated with the capture antibody (anti-mouse IL-1α and anti-mouse IL-6) in 0.05 M sodium carbonate/bicarbonate buffer (pH 9.6) overnight at 4 °C. Following thorough washing with wash buffer, non-specific protein binding sites were blocked with 5 % w/v BSA for 2 hours at room temperature. The plate was then incubated with the supernatants for 1 hour. Calibrator solutions containing 8 pg/mL to 1 ng/mL of each antigen was included to generate the respective standard curves. Following thorough washing with wash buffer, the plate was incubated for 1 hour with the biotinylated detection antibodies, then rinsed and incubated with avidin-HRP conjugate for 30 min. Signals were developed using OPD for 20 min and read at 450 nm (Epoch microplate spectrophotometer, BioTeK, Potton, UK).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.04 (GraphPad Software, La Jolla, CA, USA).Statistical significance between groups was determined using a two-tailed, two-sample t test, at a significance level of $\alpha = 0.05$.

Results and discussion

Device fabrication and characterisation

The PLA microneedle arrays had high structural fidelity to the master template (Figure 1a–f). Each array carried 35 microneedles, arranged in 2 rows of 5, 2 rows of 8, and 1 row of 9 microneedles. Microneedles within the same row were 1 mm apart, whereas between rows each microneedle was 1.2 mm from the nearest neighbour. The microneedles were essentially conical but with a slightly convex lateral surface. The height of each microneedle was 1 mm. The base radius was 115 µm.

The PLA microneedles perforated mouse skin with ease both before and after surface activation and antibody immobilisation. Skin penetration was readily evident since, when removing the microneedle array from the tissue, the skin transiently lifted. To visualise the perforations, skin samples were stained with methylene blue, a hydrophilic dye. Methylene blue selectively stained perforations in the hydrophobic stratum corneum, where the underlying hydrophilic tissue had been exposed. This produced distinct patterns on the skin that reflected the spatial arrangement of microneedles on the array (Figure 1g).

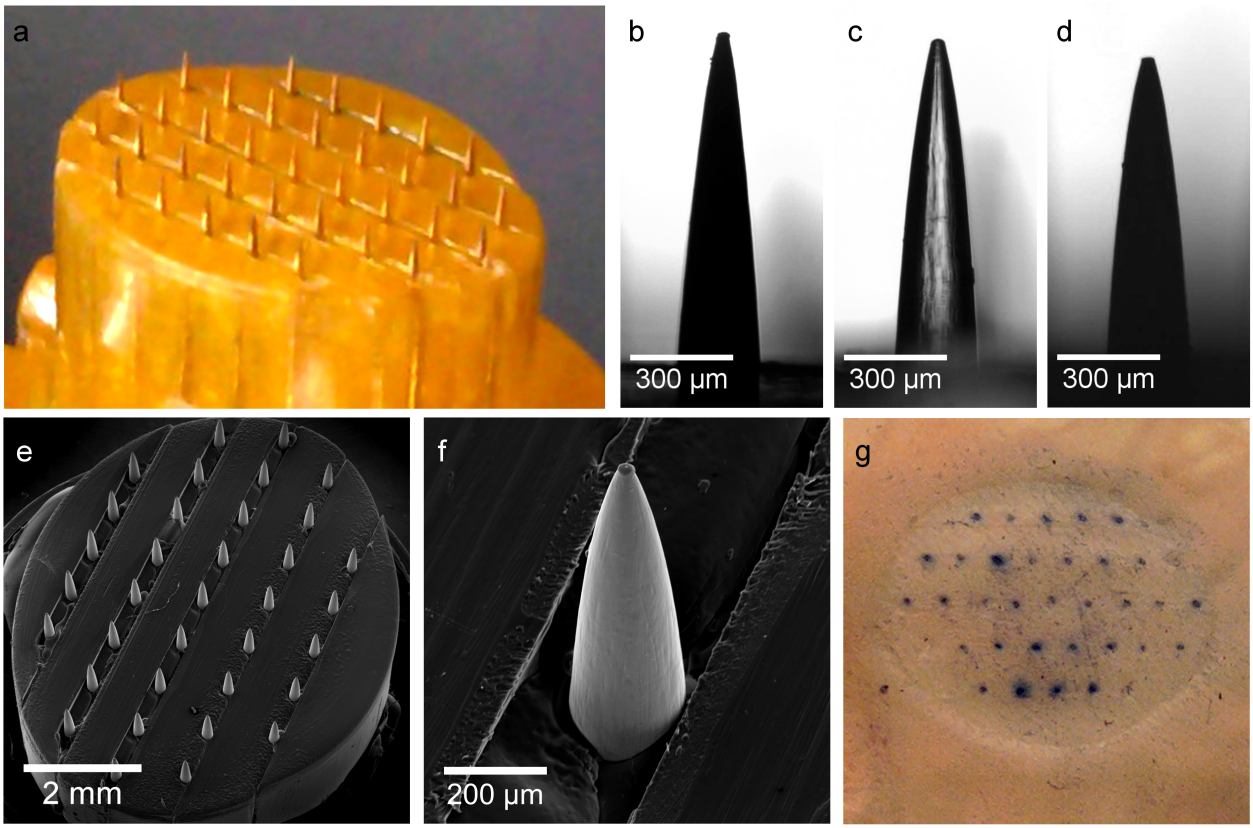

Figure 1: (a) A photograph of the template microneedle array. (b–d) Light micrographs showing: (b) a template microneedle, (c) a PLA microneedle prior to surface activation, and (d) a surface-activated PLA microneedle. (e) A surface-activated PLA microneedle array under SEM; a single microneedle on this array is enlarged and shown in (f). (g) Perforation marks on hairless mouse skin, visualised by methylene blue staining.

Antibody immobilisation

It was anticipated that PLA microneedles would not readily bind capture antibodies, due to a lack of surface functional groups. They were therefore chemically modified, by aminolysing the PLA chains with HMDA. This provided primary amine residues on the microneedle surface to covalently react with glutaraldehyde, in turn, leaving reactive aldehyde groups on the polymer surface [23]. Surface activation significantly increased antibody immobilisation to the PLA microneedles, as evidenced by the yellow colouration that developed in the substrate (OPD) solution and the greatly enhanced absorbance it produced compared with non-activated microneedles (Figure 2). Without surface activation, the substrate solution typically remained colourless for the duration of the experiment. Surface activation was also required to enable covalent attachment of the capture antibody, in order to minimise the risk of the antibodies detaching from the microneedles during or following insertion in the skin.

To our knowledge, this study is the first to describe an immunodiagnostic microneedle device based on PLA. Similar devices reported in the literature include silicon microneedles, produced by reactive ion etching [10, 11, 12, 14], and polycarbonate microneedles [13], produced by compression moulding. PLA microneedles are advantageous because they are inexpensive and simple to fabricate by micromoulding using relatively modest technology. Surface activation for PLA microneedles for antibody immobilisation requires fewer steps and much shorter incubation times (2–3 hours) compared to their silicon and polycarbonate counterparts (typically 1–2 days) [10, 11, 12, 13, 15]. Consequently, in this study, it was possible to complete antibody immobilisation and blocking of non-specific binding sites within 24 hours.

Performance characteristics

Antigen capture by microneedle devices was tested in aqueous antigen solutions and in mouse skin. Mono- and multiplex devices were tested separately because the different antibody immobilisation protocols used were anticipated to produce different performance characteristics.

Monoplex devices: antigen specificity and sensitivity

After incubating monoplex microneedle devices with antigen solutions (mouse IL-6) ranging from 0.8 pg/mL to 0.1 μg/mL and measuring the signals spectrophotometrically, the semi-log plot of absorbance versus antigen concentration generated a sigmoidal profile typical of solid-phase enzyme immunoassays (Figure 3). Thus, in principle, quantitative analysis of captured antigens using the microneedle devices is feasible. The concentration-signal profile also revealed that antigen capture was highly specific and therefore most probably mediated by the immobilised capture antibody. However, the signal produced using this detection method was relatively low (absorbance < 0.3 from 25 microneedles), albeit unambiguous. This was probably because the oxidation product (2,3-diaminophenazine) of OPD, which gave the substrate solution its yellow colour, was dispersed throughout a relatively large volume (200 μL) of liquid medium. An alternative simple and rapid detection method was therefore developed to blot the signal on a piece of paper. This concentrated the colouration at points of contact between the microneedle tips and the paper (Figure 4a), generating intense signals even from very low antigen concentrations. The sensitivity of the approach was demonstrated by incubating the device with solutions of a model antigen (human TNF-α) ranging from 50 pg/mL to 500 ng/mL. Figure 4b shows the resulting blot patterns, which corresponded to the positions of the microneedles on the array. The antigen was detected at all concentrations tested. The limit of detection was therefore $\leq$50 pg/mL.

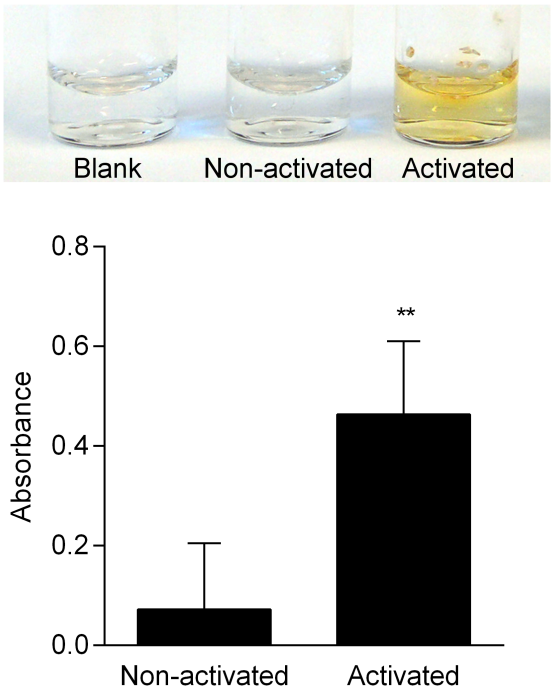

Figure 2: Antibody immobilisation on to PLA microneedles was confirmed by immunochemistry, using HRP, which produced yellow colouration in the OPD solution, the absorbance of which was measured by UV/Vis spectrophotometry. Surface activation significantly increased antibody immobilisation ($^{**}p < 0.01$), as evidenced by the colour intensity and enhanced absorbance compared to non-activated microneedles. Data are mean ± standard deviation ($n = 4$).

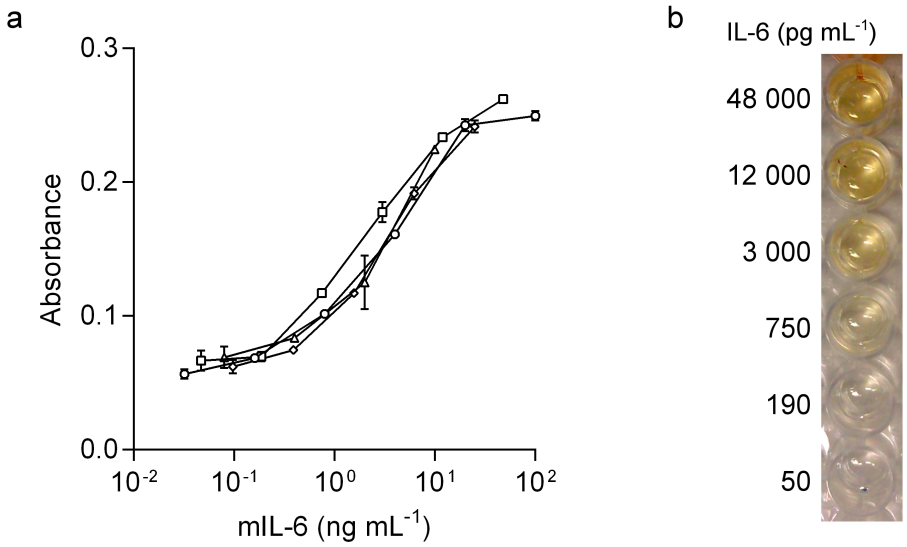

Figure 3: (a) Standard curves ($n = 4$) for mouse IL-6 generated using a monoplex microneedle device, showing the typical sigmoidal concentration-signal profile of solid-phase enzyme immunoassays. Data points are mean ± standard deviation of duplicate readings. (b) Photograph of substrate solutions corresponding to one of the standard curves shown in (a), exemplifying the decreasing signal intensity observed with decreasing mouse IL-6 concentration.

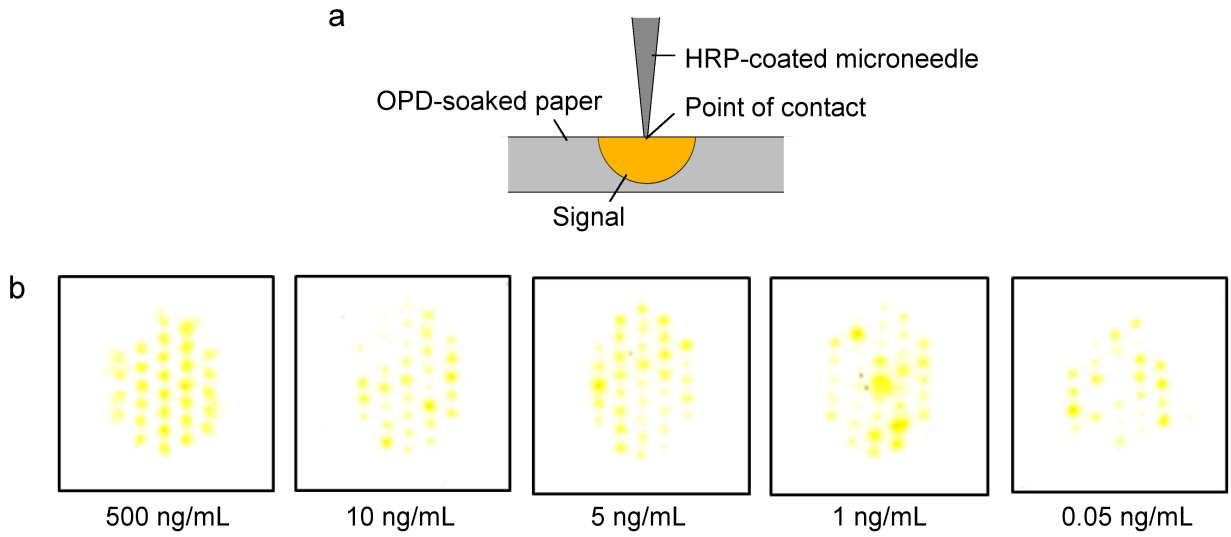

Figure 4: (a) Diagram depicting signal generation in the blotting technique, at points of contact between a microneedle tip carrying HRP and paper wetted with OPD. (b) Blot patterns showing the signal generated using this method from solutions containing various concentrations of a model antigen (human TNF-α).

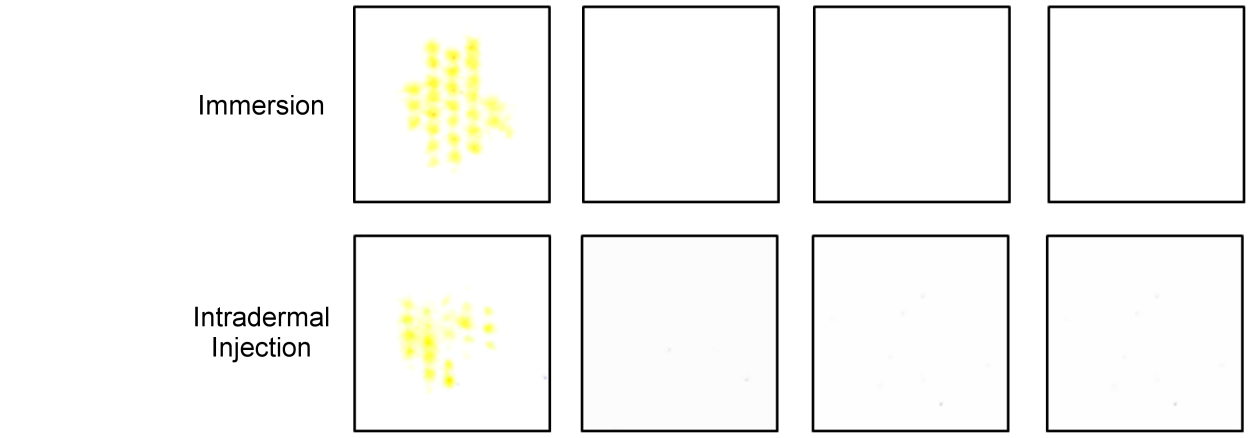

Figure 5: Antigen specificity of monoplex microneedle devices, using human TNF-α as the target antigen, and signal detection by the blotting technique. The microneedle device was either immersed in the test solution containing recombinant human TNF-α, or applied on to mouse skin into which the test solution had been injected intradermally. Control devices were set up whereby either the capture antibody or the detection antibody was replaced with an isotype known not to recognise human TNF-α, or the target antigen was omitted from the antigen solution. A signal was detected only when the corresponding TNF-α antibodies were used and when TNF-α was present in the test solution.

The blotting technique was used to re-affirm the antigen specificity of the monoplex microneedle devices. Figure 5 demonstrates that the devices were highly antigen-specific, as replacing either the capture or detection antibody with an isotype, or omitting the target antigen from the test solution, did not produce any signal. This was the case with both the device immersed in the test solution (100 ng/mL), and with the device administered on to mouse skin into which the test solution (1 μg/mL) had been injected intradermally. In the latter, an exogenous antigen (human TNF-α) was artificially introduced (rather than targeting an endogenous mouse antigen) to ensure that the target antigen was present at a detectable level. The concentration of human TNF-α injected, at 1 μg/mL, was well above the limit of detection according to previous experiments.

Comparing the sensitivity of the two detection methods, assuming both had comparable antigen binding and enzyme kinetics, the blotting method appeared more sensitive than the spectrophotometric method. The linear slope of the standard curves generally started in the region of 0.1–1 ng/mL of antigen, and the yellow colouration was typically not visually discernible below 200 pg/mL. Conversely, the blotting method produced clear signals at concentrations as low as 50 pg/mL. It was surmised that a more intense colour would tend to develop from a given amount of enzyme activity with the blotting method compared to the spectrophotometric method, because the coloured oxidation product of OPD would concentrate at the microneedle tips rather than be dispersed throughout a large volume of solution. However, the blots did not always display signals from all microneedles on a given array (Figures 4–5). This was ascribed to poor contact between some microneedle tips and the blot paper, and was overcome in later experiments (Fig. 7–9) by applying a constant downward force on the device to ensure good contact.

Monoplex devices: 30-day functional stability

Following 30 days of desiccated storage at room temperature or at 4 °C, the microneedle devices detected mouse IL-6 in aqueous solutions, producing clearly visible colour signals. However, they clearly lost some sensitivity, retaining only ~40 % of the signal produced by freshly coated devices (Figure 6). This decline is unsurprising, given that solid-phase immobilisation stabilises antibodies but drying impairs subsequent antigen binding by surface denaturation, even in the presence of protectants such as sucrose and lactose [24]. However, commercially available pre-coated ELISA plates, which are commonly dried in a similar manner with the inclusion of suitable protectants, usually have a recommended shelf life of up to 6 months. Others have reported 95 % retention of capture antibody activity in solid-phase immunoassays after desiccated storage for a year [25], suggesting that the devices can be optimised for stability. It is notable that storage temperature did not differentially affect functional stability of the devices over the course of 1 month. This suggests that the decline in signal may not be due primarily to degradation of the capture antibodies in response to heat. Other factors such as drying-induced conformational change, or antibody detachment from the microneedle surface, may be responsible for the functional decline.

Multiplex devices: antigen specificity, sensitivity and detection of endogenous skin antigens

Initially, duplex devices targeting human TNF-α and mouse IL-6 detected both antigens in solution, producing characteristic blot patterns in accordance with the composition of the solution (Figure 7). The results demonstrated the principle of multiplex detection using the devices, made possible by the remarkable antigen specificity of the approach. These findings established the principle that a device carrying any number of microneedles may be configured to detect numerous and varied antigens. However, in such systems, it is clearly necessary to dedicate some of the microneedles as

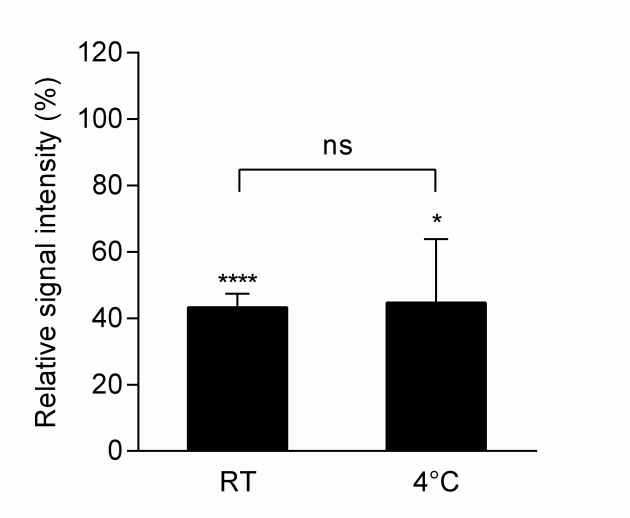

Figure 6: Stability of microneedle devices over 30 days of desiccated storage, at room temperature (RT) or 4 °C. Upon incubation with 100 ng/mL of mouse IL-6, devices that had been stored for 30 days retained ~40 % of the signal relative to that attained by freshly coated devices. Data are mean ± standard deviation ($n = 4$). ****$p < 0.0001$, *$p < 0.05$ compared to freshly coated devices (100 %) by one-sample t-test; ns: not statistically significant (two-sample t-test).

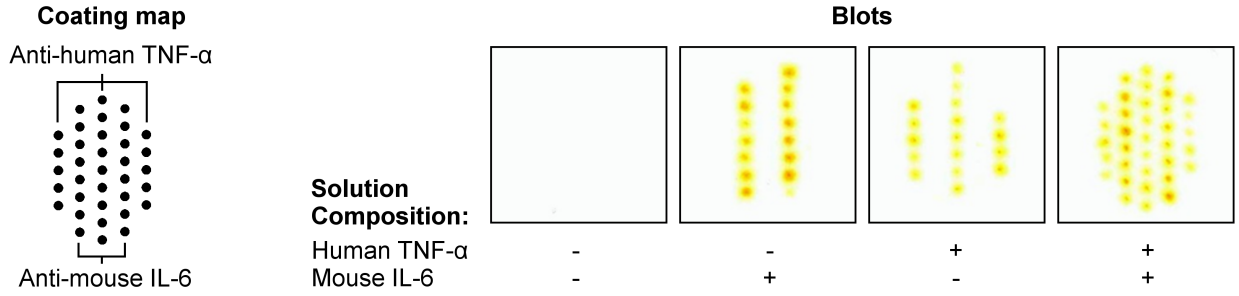

Figure 7: Antigen specificity of multiplex microneedle devices, as demonstrated by the detection of human TNF-α and mouse IL-6 using a duplex microneedle device in solutions containing either/neither/both target antigens.

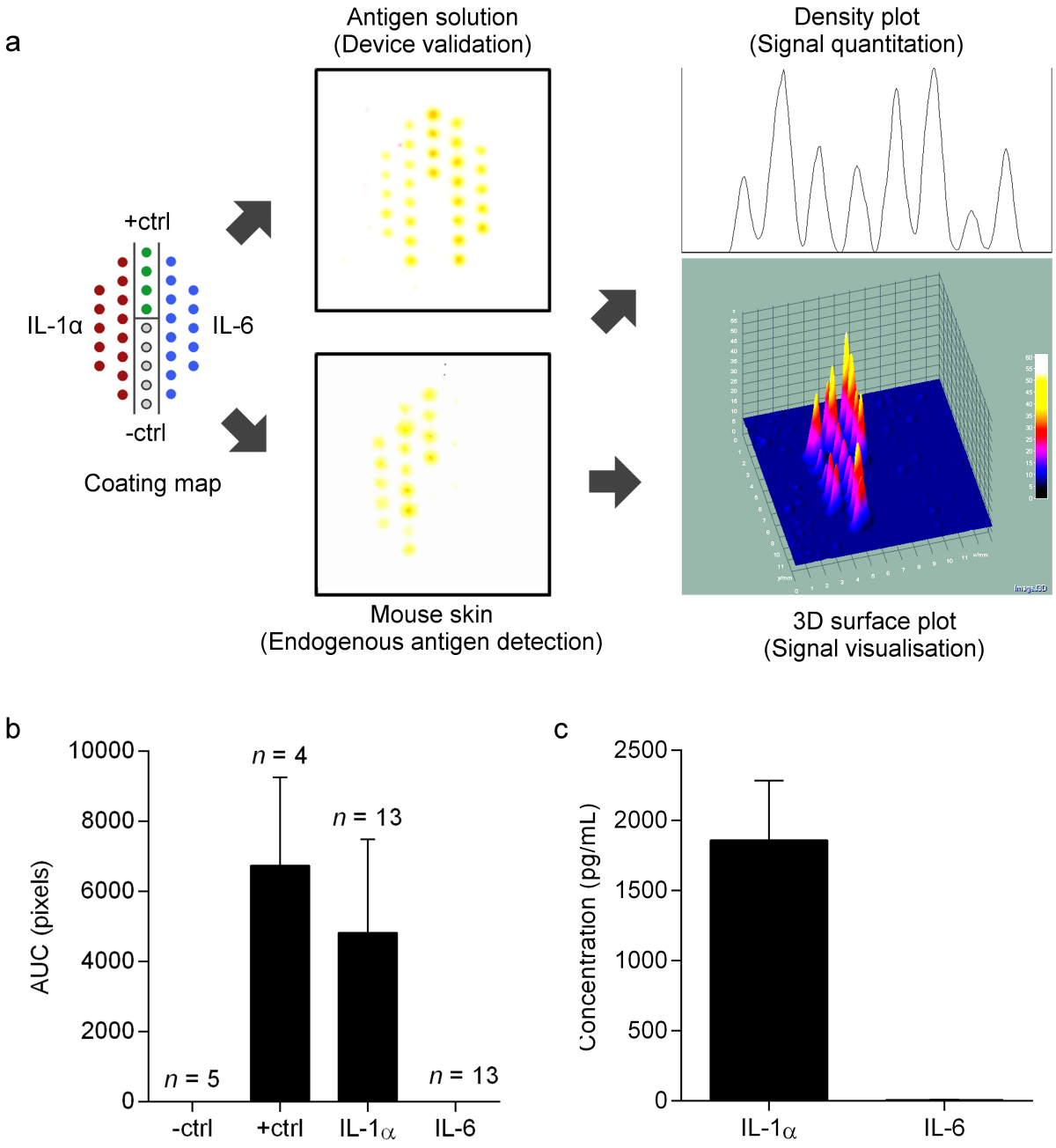

Figure 8: (a) Coating map of a quadruplex microneedle device depicting the locations of assay controls and where target antigens were expected to be detected (left), blots obtained from the device in antigen solutions and in mouse skin (centre), signal quantitation and visualisation using a density plot and a 3D surface plot, respectively (right). (b) Signal size as determined by densitometric analysis (mean ± standard deviation, representative data from a quadruplex device). (c) Cytokine content in mouse skin, as determined by conventional ELISA of skin homogenates (mean ± standard deviation; $n = 4$).

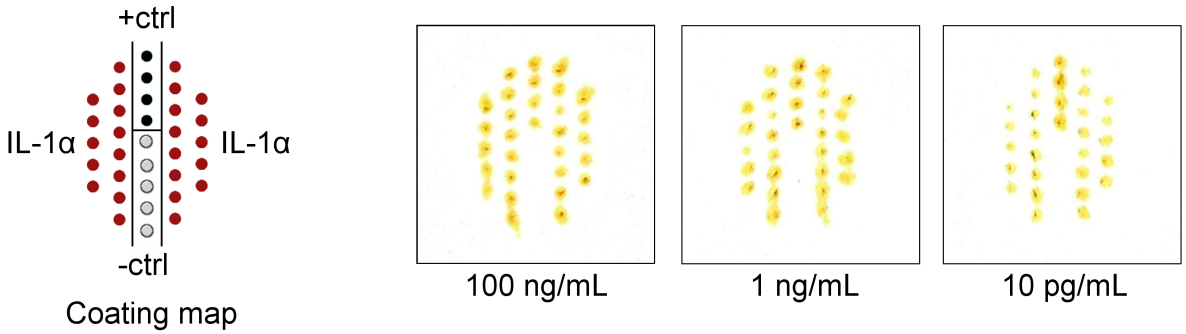

Figure 9: Detection of mouse IL-1α in solutions ranging from 0.01–100 ng/mL using a multiplex microneedle device.

internal controls. A quadruplex device was therefore designed to detect endogenous IL-1α and IL-6 simultaneously in mouse skin, whilst also incorporating positive and negative controls for validation. In this device, microneedles coated with a biotinylated antibody served as the positive control, whilst microneedles coated with a non-biotinylated antibody raised against a human antigen (human TNF-α) served as the negative control. The device detected IL-1α but not IL-6 in mouse skin (Figure 8a). Using densitometric analysis, the mean signal intensity for each target antigen was determined and found to be in agreement with antigen concentration in skin homogenates as determined by plate ELISA (Figure 8b-c). Furthermore, the device exhibited excellent sensitivity and was able to detect IL-1α in solutions at 10 pg/mL (the lowest concentration tested; Figure 9), close to the limit of detection for this antigen with plate ELISA.

Benefits and limitations

The blotting method allows rapid (near real time) visualisation for the presence of specific antigens, but is evidently qualitative though with high sensitivity. Quantitative analysis is possible with computerised densitometry, as demonstrated in Fig. 8. However, further work is needed to establish the concentration-signal relationship in this format. To this end, a number of variables such as capture antibody coating density/uniformity and incubation time will need to be optimised. Moreover, the blotting method generates signals only at microneedle tips, not from the full length of the microneedles. Consequently, the device is able to detect antigens captured at a specific skin depth, whilst ignoring antigens captured from other skin layers. The sampling depth is dependent on the length of the microneedles, and may be adjusted by varying microneedle length. This can be advantageous in applications where the depth at which a biomarker is detected needs to be clearly defined, such as in constructing depth profiles for skin biomarkers.

Previous microneedle-based biomarker extraction studies have necessitated either dilution of the signal in a liquid medium [11, 12, 13, 14, 19] or direct quantification of fluorescence on the microneedles [10, 15] for detection. Signal dilution is undesirable as it inevitably reduces the sensitivity of the test, especially for biomarkers that are present at very low levels. On the other hand, fluorescence is susceptible to photobleaching or quenching [26, 27], resulting in weak or transient signals. These previous methods also require specialist laboratory equipment such as a confocal laser scanner and microplate reader. In contrast, the blotting method developed here requires no specialist imaging equipment and is not subject to photobleaching or quenching. The primary test result is a physical, persistent blot visible to the naked eye. This technique will be

particularly useful as a binary classification test, where the presence or absence of multiple antigens can be quickly determined from the blot patterns without further processing. For more quantitative analysis, digital images of the blots can be captured using a standard office scanner, and the density of the blots measured using free and open-source software (ImageJ). Together, these advantages are likely to significantly reduce the cost and increase the accessibility of the test especially in resource-limited settings.

The results of this study demonstrate that multiplex detection of specific antigens is achievable with the PLA microneedle devices. *In situ* antigen capture has the potential to deliver significant savings in time, cost and labour in clinical diagnosis. For example, a typical test took about 5 hours from the point of device administration to signal detection, compared to days and even weeks for histological tests of biopsies. The savings are achieved by removing the need for extensive histological processing such as sample fixation, embedding, sectioning and slide preparation otherwise necessitated by histochemical analysis. Multiplex designs enable further savings by analysing multiple antigens simultaneously. However, microneedles are known to elicit local skin reactions, such as transient erythema and increased blood flow [28], indicative of momentary changes in skin protein profile. For example, local expression of some cytokines, including IL-1β and TNF-α, was elevated 6 hours after microneedle administration [29]. It is not clear whether significant microneedle-induced local protein expression would occur within the much shorter time frame ($\leq$1 hour) required for our antigen extraction and the extent of any such interference with antigen detection. Nonetheless, care needs to be exercised in biomarker selection and assay development to account for possible biomarker induction by microneedles.

Conclusions

In this study, a series of monoplex and multiplex immunodiagnostic microneedle arrays were designed, constructed, validated and tested. Cytokines were extracted from skin tissue and analysed using a novel blotting method that can be coupled with computerised densitometry to enable signal quantitation. The novel blotting method detected antigens captured at the microneedle tips, and can therefore facilitate biomarker analysis at a specific skin depth. The microneedle devices exhibited high antigen specificity and sensitivity, but had reduced functionality after 30 days in desiccated storage. The tests were rapid, producing results within 5 hours of device administration. Furthermore, multiplex microneedle arrays were able to detect multiple antigens simultaneously, whilst also incorporating experimental controls for result validation. This approach of antigen detection can lead to significant savings in time, cost and labour compared to histological methods.

Acknowledgements

The authors thank the staff at the Centre for Advanced Microscopy (University of Reading) for their help with SEM experiments, and staff at the Bioresource Unit (University of Brighton) for donating cadaver mouse skin samples. Conflict of interest KWN, WML and ACW declare that they have no conflict of interest.

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The final publication is available at Springer via
http://dx.doi.org/10.1007/s13346-015-0231-5.