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Surface-modified microprojection arrays for intradermal biomarker capture, with low non-specific protein binding

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Minimally invasive biosensors are of great interest for rapid detection of disease biomarkers for diagnostic screening at the point-of-care. Here we introduce a device which extracts disease-specific biomarkers directly from the upper dermis, without the needle and syringe or resource-intensive blood processing. Using antigen-specific antibodies raised in mice as a model system, we confirm the analytical specificity and sensitivity of the antibody capture and extraction in comparison to the conventional methods based on needle/syringe blood draw followed by processing and antigen-specific ELISAs.

New technologies are urgently required to translate biomarker discovery research into practical molecular diagnostic assays for population-wide screening and early disease detection.[1,2] Blood is currently the most important clinical sample.[3] However, while assay chemistries and detection strategies are continually evolving, blood samples are still mostly collected with the needle/syringe—which is invasive, requires specialist training and is not ideal for routine monitoring and screening,[4] especially in resource-poor areas.

Recently, densely packed nano- and micropillar arrays have been incorporated into microfluidic devices to enhance biomolecular capture and separation from bulk fluids. The enhanced probe capture from blood is achieved by increasing surface area of capture probes and improving kinetics by elevating biomolecular reactions above the flat surface.[5] While such devices have been used to extract DNA and other species from whole blood, they still rely on blood samples first being drawn from the body—primarily by the needle/syringe.

Here, we introduce an array of significantly larger sharpened micropillars (termed microprojection arrays (MPAs)) which we tailor, both chemically and mechanically, for the selective extraction of biomarkers directly from serum-rich fluids in the skin epithelia. By applying these devices to the skin, we breach the tough outer skin layers to target the upper dermis[6,7] in a pain-free manner,[8] in order to directly capture and remove specific biomarkers. Related devices with epi/dermal targeting have been used for vaccine delivery,[9] also hollow microneedle devices have been adapted for glucose testing.[10,11] However, to our knowledge, this is the first published work on a device designed to capture specific circulating biomarkers present in dermal fluid in a pain-free manner.

MPAs were synthesised from (100) silicon using a process of Deep Reactive Ion Etching (DRIE) in Rutherford Appleton Laboratory, Oxford, UK.[6,7] Scanning electron microscopy (SEM) of gold-coated silicon MPA devices revealed a dense array (20 000 cm^{-2}) of sharp microprojections ~65 μm in length, 25 μm in base diameter, tapering to sub-micrometer sharp tips (Fig. 1(a)–(c)). With this geometry, we applied the devices to C57BL/6 mouse ear skin at a constant application velocity,[6] achieving reproducible penetration targeted to the

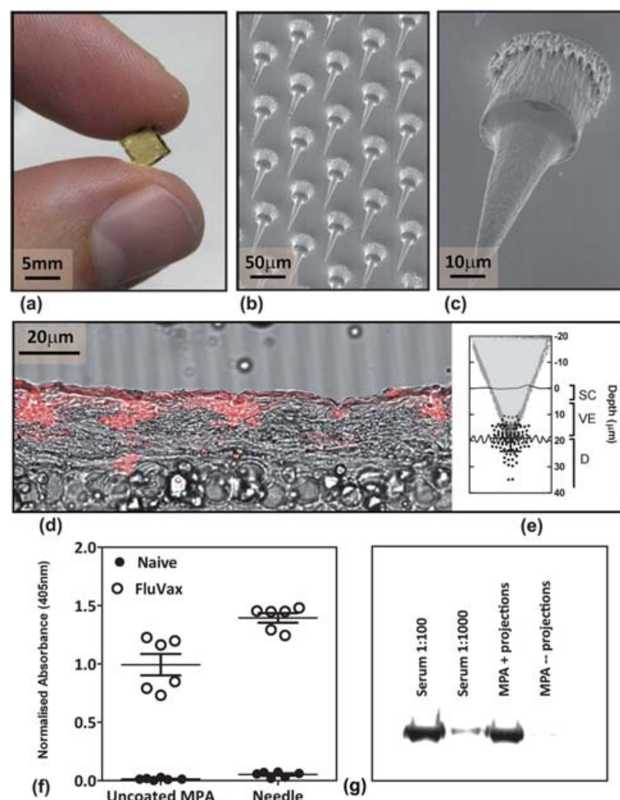

Fig. 1 Microprojection arrays (MPAs) penetrate through the skin's outer layers to sample fluid in the upper dermis; (a) photograph of MPA showing size relative to human fingers; (b and c) SEM images showing a highly dense array of sharp microprojections; (d) application of MPA coated with a fluorescent dye shows (e) dermal penetration at 19 ± 5 μm; (f) AF-IgG biomarker specifically detected in FluVax®-vaccinated animals by either MPA or standard needle/syringe; (g) Western blot shows that flat wafers (−) applied to skin extract no detectable albumin in comparison to regular MPAs (+). Note: SC = stratum corneum; VE = viable epidermis; D = dermis.

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upper dermis (19 ± 5 μm, Fig. 1(d) and (e)) which is rich in capillary blood.[12]

Prior to any surface modifications, we hypothesised that MPAs penetrating into the dermis should extract serum-rich fluid containing systemic biomarkers. To test this, we vaccinated mice with FluVax® by intramuscular injection and measured the resultant anti-FluVax®-IgG (AF-IgG) response after 21 days.[9] As shown in Fig. 1(f), analysis of MPA-extracted fluid and needle-extracted blood revealed comparable AF-IgG levels by ELISA.[13] Furthermore, we confirmed that the microprojections were specifically required for protein capture, while flat devices could not bind albumin when applied to the skin using the same method (Fig. 1(g)).

We then grafted a hetero-bifunctional PEG (Creative PEGworks, NC, USA, Cat# PBL-8076, M_w = 5000 g mol^{-1}) to the gold-coated microprojections (herein referred to as "PEG devices") to both reduce non-specific protein adsorption and provide an anchor point for capturing protein attachment (Fig. 2(a)). X-Ray Photoelectron Spectroscopy (XPS) analysis confirmed the molecular composition of the MPA surfaces before and after modification (Fig. 2). Uncoated MPAs (Fig. 2(b)) showed a typical gold surface (Au4f, 84 eV) with a small degree of aliphatic carbon contamination (C1s, 284.6 eV). Upon chemisorption of PEG chains to the gold surface *via* the thiol group (under "cloud-point" condition—5 μM in 0.6 M K_2SO_4 at 60 °C in a water bath overnight[14]), a characteristic ether peak was observed at 286 eV (Fig. 2(b)). We did not observe thiol (S2p) or carboxy (C1s) peaks. We assert this is because the C–S : C–O and C=O : C–O ratios were ~1 : 100, or less than 0.2% and thus undetectable by XPS. By comparing the coated and uncoated intensities of Au4f7/2 gold peaks after ion beam etching, we calculated layer thickness of ~4.6 ± 1.0 nm using Beer's law, suggesting a grafting density of ~0.6 ± 0.1 PEG nm^{-2}—indicating a highly dense surface coating.[15] The standard deviation across an individual MPA surface (estimated by analysing three separate parts of each device) was similar to that between batches coated on different days, indicating consistent surface coverage.

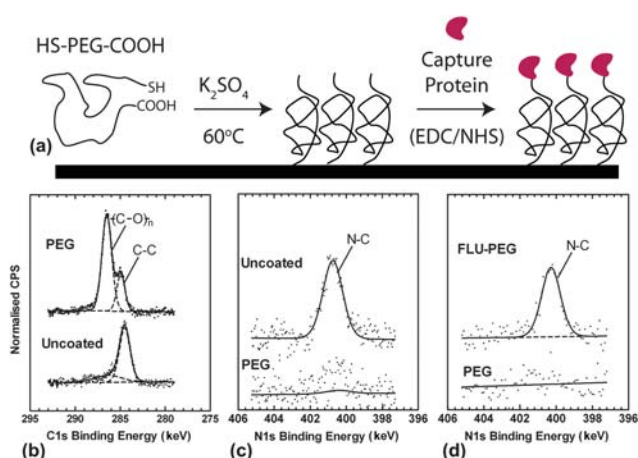

Fig. 2 Surface modification strategy for specific biomarker capture. Scheme (a) shows the process of coupling PEG and capture proteins to the MPA surface; XPS high resolution spectra of (b) C1s region before/after PEG coating; (c) N1s region showing effect of PEG coating on 100% serum adsorption; (d) N1s spectra showing attachment of capture protein to PEG layer *via* EDC/NHS chemistry.

To determine the effect of PEG coating on protein adsorption, we applied coated and uncoated MPAs to mouse skin for 10 minutes (time used throughout the study) and then rinsed thoroughly with PBS/0.1% Tween to remove loosely bound species. Fig. 2(c) shows >90% reduction in protein adsorption with PEG coating—a conservative estimate considering the variability of the N1s "noise" level observed for PEG-coated MPAs. To complete the surface modification for AF-IgG testing, AF-IgG capture proteins (FluVax® [a commercial trivalent influenza vaccine] *via* standard EDC chemistry[16]) were then grafted to the carboxyl terminus of the PEG group (termed "PEG–FLU" or "PEG–OVA" device in the case of the negative control), resulting in the appearance of a characteristic amide peak at 399 eV (Fig. 2(d)).

With our PEG–FLU surface modifications confirmed, we tested the devices *in vitro* for specific binding of AF-IgG in 10% mouse serum. We designed a simple detection strategy ("MPA-ELISA" assay[17]) whereby after serum incubation, MPAs were washed to remove loosely bound species and then an A647-labelled detection antibody was added to bind specifically to captured AF-IgG, and then quantified.[18,19] Comparison between naïve and Fluvax®-immunized serum binding to surface modified MPAs was used to determine the specificity of AF-IgG capture (Fig. 3(a)). PEG–FLU MPAs incubated with Fluvax®-immunized mouse serum for 10 minutes showed significantly higher fluorescence intensity per device (p-value = 0.017, signal/noise = 16) in comparison to the same

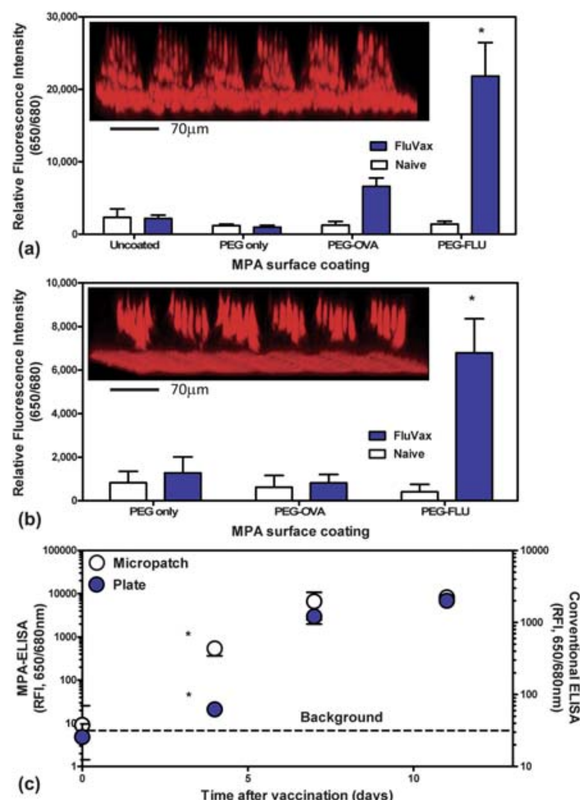

Fig. 3 Fluorescent MPA-ELISA results for AF-IgG detection. (a) AF-IgG binding to different surface-modified MPAs in serum; (b) AF-IgG binding to different surface-modified MPAs in skin; (c) comparison between MPA-ELISA and standard ELISA involving serum analysis after needle/syringe whole blood extraction. Insets show confocal microscope 3D images of MPA devices after application to serum or skin.

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devices exposed to naïve serum. Similarly, PEG–FLU MPAs also showed significantly higher signals in comparison to uncoated (p-value = 0.018), PEG (p-value = 0.016) or PEG–OVA MPAs (p-value = 0.032), regardless of the source of serum.

We used the same Fluvax®-vaccination model to test the MPA-ELISA assay *in vivo*, applying devices directly to mouse ear skin for 10 minutes. Results shown in Fig. 3(b) were similar to those for incubation in extracted serum. The PEG–FLU-coated MPAs extracted significantly more AF-IgG from immunized mice than naïve (p-value = 0.0286, signal/noise = 16) and also with respect to either PEG-coated (p-value = 0.0286) or ovalbumin-coated devices (p-value = 0.0286). These data confirm high analytical specificity. There was no significant difference between naïve and Fluvax®-immunized mice for either PEG-coated (p-value = 0.4857) or PEG–OVA-coated MPAs (p-value = 0.3429), demonstrating that the non-specific binding properties of the PEG layer were maintained even after ovalbumin protein attachment. Importantly, naïve animals did not mount a detectable antibody immune response following PEG–FLU MPA application (data not shown).

In comparison to the serum incubation results, skin application yielded a different fluorescence distribution along the microprojections, as analysed by confocal microscopy (Fig. 3(a) and (b) insets; LSM510 Meta, Carl Zeiss, Australia). First, the serum incubation resulted in relatively even distribution (Fig. 3(a) inset)—which is consistent with thorough mixing of the serum on the immersed patch surface. In contrast, skin application resulted in a tip-specific fluorescence signal (Fig. 3(b) inset) which was consistent with 40–50% of the microprojection length penetrated into the skin. In both cases, we could not avoid some reflection from the excitation source off the base of the MPAs, visible most clearly in the inset of Fig. 3(b).

With high analytical specificity established, the next step was to determine the analytical sensitivity of the MPA device—and to directly compare the assay to a current diagnostic standard. To do this, we measured the AF-IgG levels using both MPA-ELISA and conventional ELISA at time points, prior to peak post-vaccination IgG levels (*i.e.* days 0, 4, 7, 10, and 15). To ensure comparative results, we used the same incubation times (10 minute serum per skin, 30 minutes detection antibody) and reagents. We hypothesized that by comparing the time taken to reach a detectable signal—using both methods—we could draw conclusions about their relative sensitivities. Pre-immunized animals (*i.e.* day 0) registered signals equivalent to assay background in both cases. Over the following days, both techniques registered increasing AF-IgG levels, reaching a plateau at approximately 12–15 days following vaccination. Significantly, both methods measured statistically detectable levels of AF-IgG after day 4 (p-value = 0.0286, corresponding to 30 ng mL^{-1} of anti-hemagglutinin antibody [Abcam, MA, USA, ab26228] from a standard curve), indicating equivalent analytical sensitivities between the two methods.

In this study, we show that a systemic biomarker is selectively removed from the dermis of live mice for subsequent detection in a minimally invasive manner. Importantly, negligible sample processing was required between skin application and detection of antibody binding. This is in stark contrast to current methods, whereby after extraction of a large volume of whole blood using a needle/syringe, the sample needs to be clotted, separated from solids by centrifugation and often stored under controlled conditions prior to analysis. It is not surprising that standard processing steps result in systematic errors in biomarker concentration.[19,20] However, at the same time, a current limitation with MPAs is that we have only shown sampling of soluble markers, which includes those present in serum (proteins, DNA, small molecules). We have not yet examined intracellular species at this stage.

Indeed, we foresee several aspects of our new diagnostic tool warranting further investigation. First, as yet, we have not identified the exact fluid sources sampled by MPAs. We know the microprojections penetrate into the upper dermis—where common blood products were successfully sampled. With the projection surface area in contact with both the viable epidermis and upper dermis, it seems reasonable to assert that a mixture of blood and interstitial fluid is sampled. Further proteomic/genomic investigations are ongoing for confirmation, which may be important in the context of systemic *versus* local tissue diseases. Secondly, the application time on the skin is also an important variable requiring investigation. In some instances (*e.g.* pediatric monitoring) fast application times would be preferable; especially for ease-of-use and patient compliance. This will need to be considered against longer application times of MPAs (*e.g.* hours or days), which might open up the sensitive detection of rare biomarkers. Thirdly, multiplexed detection of several biomarker species on the same device would provide greater clinical specificity for diagnostic purposes. One of these biomarkers could be an "internal control" (*e.g.* albumin, haemoglobin, and IgG) which could then facilitate relative quantitation between skin sites of varying thickness, depth, *etc.* Finally, increasing the penetration depth of the MPA microprojections will be important for human application. Both application velocity[6] and microprojection length[21] can be further increased to meet the challenge of targeting the human dermal papillae at 58–65 μm below the skin surface (at the volar forearm).[22]

In summary, the MPA technology has the potential to provide a practical alternative approach to blood sampling, enabling more widespread use of assays designed for early detection and routine disease screening based on biomarker analysis.

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16 MPAs were rinsed and resuspended in 100 μL water in preparation for EDC-mediated protein crosslinking. 10 μL of both 50 mg mL^{-1} EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and 50 mg mL^{-1} NHS (*N*-hydroxysuccinimide, Thermo Scientific) were added and the samples were agitated for 20 minutes. After washing in 0.1 M MES buffer (2-(*N*-morpholino)ethanesulfonic acid, ICN Biomedical), MPAs were suspended in 100 μL 0.1 M MES and 500 μL FluVax® 2008 (CSL Ltd.) or ovalbumin (Sigma) solution (0.2–20 μg) was added. Samples were agitated at room temperature for two hours and rinsed in PBS/0.1% Tween. The coating procedure was performed twice to ensure maximal coating.
17 MPA-ELISA assays were carried out after serum incubation or skin application to measure the relative amount of AF-IgG binding to MPA devices using a confocal fluorescent scanner (LS Reloaded Confocal Scanner, Tecan, Switzerland) and the average fluorescence intensity per MPA recorded using ImageJ. After thorough washing in PBST, A647-conjugated anti-mouse-IgG (Cat#A21237, Invitrogen, Australia), 1 : 600 diluted in PBST/0.1%BSA was added to the MPAs for 30 minutes before a final wash and fluorescence analysis as described above.
18 Results were plotted using GraphPad PRISM V5.03 software. Column graphs represent average and standard deviation of technical replicates in the case of serum incubation (3 MPA per data point) with *t*-tests used to determine *p*-values. For skin application experiments Mann–Whitney tests were used to determine *p*-values in order to account for biological replicates (4 mice per data point); those comparisons with significant *p*-values (5% significance level) are indicated by asterisks in the relevant figures.
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