■ RESEARCH

The development of fibronectin-functionalised hydroxyapatite coatings to improve dermal fibroblast attachment *in vitro*

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The success of long-term transcutaneous implants depends on dermal attachment to prevent downgrowth of the epithelium and infection. Hydroxyapatite (HA) coatings and fibronectin (Fn) have independently been shown to regulate fibroblast activity and improve attachment. In an attempt to enhance this phenomenon we adsorbed Fn onto HA-coated substrates. Our study was designed to test the hypothesis that adsorption of Fn onto HA produces a surface that will increase the attachment of dermal fibroblasts better than HA alone or titanium alloy controls.

Iodinated Fn was used to investigate the durability of the protein coating and a bioassay using human dermal fibroblasts was performed to assess the effects of the coating on cell attachment. Cell attachment data were compared with those for HA alone and titanium alloy controls at one, four and 24 hours. Protein attachment peaked within one hour of incubation and the maximum binding efficiency was achieved with an initial droplet of 1000 ng. We showed that after 24 hours one-fifth of the initial Fn coating remained on the substrates, and this resulted in a significant, three-, four-, and sevenfold increase in dermal fibroblast attachment strength compared to uncoated controls at one, four and 24 hours, respectively.

Conventional stump–socket prostheses are the treatment of choice for most above-knee amputees; however, a poorly fitting socket will frequently cause pressure sores, infection and an abnormal gait.[1] An intraosseous transcutaneous amputation prosthesis (ITAP) can overcome these problems[2] as well as providing improved proprioception.[3] During normal wound healing, epithelial cells at the margin of the wound migrate to re-establish the protective barrier function of the skin. Around an ITAP this results in downgrowth of the epithelium and pocket formation, which in turn provides a route whereby pathogens can enter and cause infection. For an ITAP to be successful, a tight seal at the skin–implant interface is essential.[4,5]

Deer antlers have been studied as biomimetic models of ITAP[6] and have emphasised the crucial role of dermal tissue adhesion in preventing downgrowth. Surface modifications and synthetic hydroxyapatite (HA) coatings have been incorporated into the design of ITAPs to enhance dermal adhesion,[4-7] and biological coatings have been shown to improve dermal fibroblast attachment both *in vitro* and *in vivo*.[8,9]

Cell adhesion is modulated by the extracellular matrix (ECM). The glycoprotein fibronectin (Fn) is a principal component of ECM, and it contains cell integrin-binding sequences; these include the amino acid sequence arginine-glycine-aspartic acid (RGD in one-letter amino acid code), through which it promotes cell-matrix adhesion.[10] Fn is readily adsorbed onto biomaterials, and enhances fibroblast activity[11] and attachment by upregulation of focal adhesion expression *in vitro*.[8,12] Focal adhesions are specialised electron-dense regions of the plasma membrane which create intimate, discrete contacts of 10 nm to 15 nm with the substratum of cells.[13] Quantification of the number of focal adhesions per unit cell area has been shown to be an accurate way of measuring the direct biophysical strength of dermal fibroblast attachment *in vitro*.[14]

HA is a naturally occurring mineral apatite that comprises 70% of bone.[15] Synthetic HA coating of endoprostheses promotes osseointegration,[16] and Fn functionalisation of HA-coated titanium alloy has been shown to increase dermal tissue attachment *in vivo*.[9] Despite extensive investigations into the interaction of ECM proteins with HA,[17-20] the precise nature of the interaction is not clear. This study investigated the loading and release kinetics of Fn from HA in the hope of providing new data that may enable us to establish an optimal coating regime capable of enhancing dermal fibroblast attachment. This could then be applied to ITAPs in order to enhance the interface between implant and skin. The aim of

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this study was to assess the loading, release and durability kinetics of Fn on HA substrates and to assess the effect of Fn-functionalised HA on dermal fibroblast attachment *in vitro*. We hypothesised that adsorption of Fn onto HA would produce a surface that would increase the attachment of dermal fibroblasts compared with HA alone and titanium alloy controls.

Materials and Methods

The study was divided into two parts: the first to determine the loading, release and durability kinetics of Fn coatings on HA, and the second to assess dermal fibroblast attachment to Fn-functionalised HA.

Fn loading, release and durability kinetics experiments. Disc preparation. Sintered HA discs 11.7 mm in diameter and weighing 1 g, made in 12 mm casts with 1.5 tonnes of pressure applied over 2 hours at 1250°C (5°C ramp/min) to HA powder (Apatech, Elstree, United Kingdom), were compacted in a mould and heated to 500°C. X-ray diffraction analysis (XRD) was performed to assess the crystallinity and purity of the HA; XRD patterns recorded using an X'Pert Pro Diffractometer (PANalytical Ltd, Cambridge, United Kingdom) showed that the abundance of calcium phosphate and hydroxyl groups and the crystallinity of HA were identical to those used previously *in vitro* and to the plasma-sprayed HA coating used for ITAPs *in vivo*.[9]

Fibronectin: coating and radiolabelling. Human plasma fibronectin (Fn) (F2006; Sigma-Aldrich, Gillingham, United Kingdom), diluted in phosphate-buffered saline (PBS), was used throughout the experiment. Fn was custom-labelled by PerkinElmer (Waltham, Massachusetts). A modified chloramine-T procedure was used to produce ^{125}I-Fn, which was then used to quantify the amount of Fn remaining on the HA discs. The optimal purification using high-performance liquid chromatography (HPLC) yielded 45% incorporation, > 95% purity, a concentration of 0.27 mCi/ml and a specific activity of 30 μCi/μg. ^{125}I produces both gamma and beta radiation. HA discs were coated with 50 μl droplets of Fn, which covered the entire surface of each disc to produce ^{125}I-FnHA discs. The ^{125}I-FnHA discs were rinsed with PBS three times before being used in the series of experiments described below. All techniques were performed at 21°C under sterile conditions using aseptic technique. All uncoated control surfaces were treated with equal volumes of PBS for the same period of time.

^{125}I-FnHA quantification. A Tricarb 2900TR liquid scintillation counter (PerkinElmer) was used to detect gamma radiation in counts per minute (CPM). Following three washes in sterile PBS, discs were placed face up in 5 ml scintillation tubes and immersed in 4.5 ml of scintillation fluid. QuantaSmart software (PerkinElmer) supplied with the liquid scintillation counter was used, with correction for ^{125}I half-life, at an external standard terminator of 0.5 s and a count time of 1 min. The scintillation counter was calibrated using appropriate standards and each sample was counted twice. The results of the loading and release kinetics experiments were determined from a standard calibration curve.

Optimisation of ^{125}I-Fn coating of HA. Six replicates were used for all studies unless stated otherwise.

Effect of duration and quantity on ^{125}I-Fn loading of HA. In order to investigate the optimal time to leave ^{125}I-Fn on the HA discs, 500 ng of ^{125}I-Fn was placed on the disc surfaces for zero, 0.5, one and two hours before analysis. In order to determine the maximum possible coating concentration, 100 ng, 250 ng, 500 ng, 1000 ng and 1500 ng of ^{125}I-Fn were placed on the disc surfaces for one hour.

Durability of ^{125}I-FnHA coating. As described above, 1000 ng of ^{125}I-Fn in 50 μl was added to the discs. In order to assess the durability of the ^{125}I-Fn coating, discs were immersed in fetal calf serum (FCS) (First Link Ltd, Birmingham, United Kingdom). The discs were analysed immediately after three washes in sterile PBS (time zero) before immersion in FCS and incubation at 37°C. Samples were removed and washed three times with distilled water, and the amount of ^{125}I-Fn remaining on the discs was measured at one, four, eight and 24 hours.

Dermal fibroblast attachment experiments. Disc preparation and characterisation. Surgical grade titanium alloy (Ti-6Al-4V) discs, 10 mm in diameter, were ground, polished and cleaned to the level required for orthopaedic implant manufacture, and were used as controls (Pol group). Non-Fn-functionalised sintered HA discs (HA group; manufactured as described above) were used to represent the HA that is currently used for ITAPs.[7] To assess the effects of Fn functionalisation of HA on dermal fibroblast attachment, 1000 ng of Fn were then applied to the HA discs (HAFn) for one hour, as described above. The surface roughness (R_a), mean maximum height of the profile (R_z) and mean spacing of irregularities of the profile (S_m) were measured using a Mitutoyo Surftest SV-400 Surface Profiler (Mitutoyo, Warwick, United Kingdom). Discs were sterilised in a 2100 Classic Clinical Autoclave (Prestige Medical, Blackburn, United Kingdom) for 11 minutes at 126°C and a pressure of 1.4 bar.

Dermal fibroblast culture and seeding. Fibroblasts (1BR.3.G cells, ECACC/Sigma-Aldrich) were cultured in Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich) with 4500 mg/l glucose, 1% non-essential amino acids, 1% penicillin/streptomycin (Invitrogen Corporation, Paisley, United Kingdom) and 10% FCS (First Link) at 37°C with 5% CO_2; 2500 cells per disc were seeded for one, four and 24 hours on Pol, HA and HAFn discs.

Fibroblast focal adhesion detection method. The discs were washed twice in PBS and fixed in formal saline for five minutes. Four five-minute washes in PBS were followed by incubation for two hours with a mouse monoclonal anti-human clone HUV-1 (V9131 Sigma-Aldrich = Anti-vinculin) (1:100) and Triton X-100 (1:500) in sterile PBS. After three washes in PBS the discs were incubated for 45 minutes with fluorescein isothiocyanate (FITC) conjugate in a secondary antibody solution (F2883 Sigma-Aldrich = Anti-mouse) (1:168 in sterile PBS), and then washed three times in PBS before analysis.

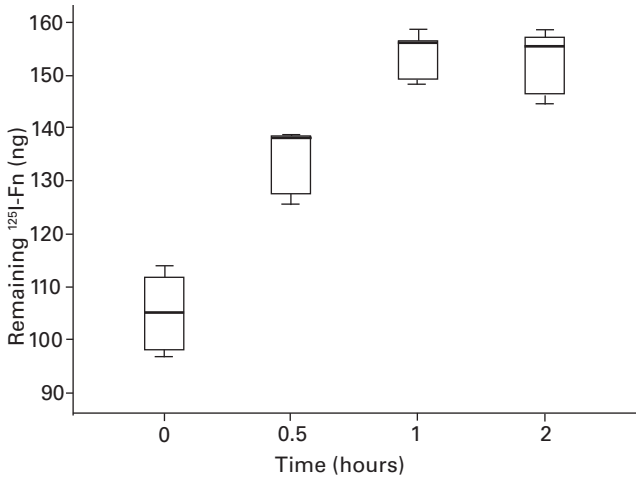

Fig. 1

Box plot showing the amount of ^{125}I-fibronectin (^{125}I-Fn) (in ng) remaining on hydroxyapatite discs after an initial loading with 500 ng, rinsing and detection (as described in text) after incubations of 0, 0.5, one and two hours. The boxes denote the median and interquartile range, and the whiskers denote the full range.

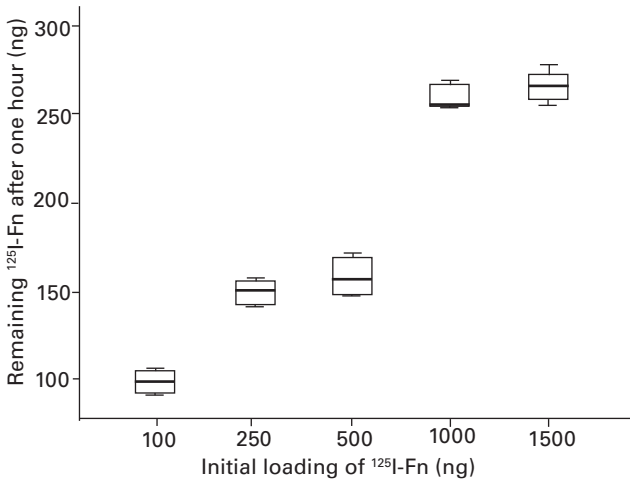

Fig. 2

Box plot showing the amount of ^{125}I-fibronectin (^{125}I-Fn) (in ng) remaining on hydroxyapatite discs after initial loading with between 100 ng and 1500 ng of Fn, incubation for one hour, and rinsing and detection (as described in text). The boxes denote the median and interquartile range, and the whiskers denote the full range.

Fibroblast focal adhesion and cell area quantification. After vinculin staining at one, four and 24 hours, focal adhesion quantification was carried out using a Carl Zeiss microscope (Carl Zeiss Ltd, Welwyn Garden City, United Kingdom) with ×50, and ×100 objective lenses. For each disc 15 cells were analysed. A random field of view was selected and the vinculin markers on the cells (defined as individually distinguishable immunofluorescent markers within the cell, and present at the cell boundaries, as previously described[8,9,14]) were manually identified and counted by two independent observers (CJP, ME) who were blinded both to the test substrate and to one another. Using kappa statistics, kappa scores indicated almost perfect inter-observer agreement (> 0.90), and so the data presented are those of both observers combined. Cell areas were measured using Axiovision Image Analysis Software (Axioimage 4.4; Carl Zeiss, Gottingen, Germany). The number of vinculin markers per unit cell area was calculated by dividing the number of vinculin counts by the cell area.

Statistical analyses. The data did not fit the assumptions required for parametric testing and were analysed using Mann-Whitney U tests to compare medians. Box plots showing median values, whole and interquartile ranges, and median values were expressed with 95% confidence intervals (CI). All numerical data are stated as median values (with 95% CI) unless otherwise stated. Results were considered significant when the p-value ≤ 0.05.

Results

Fn loading, release and durability kinetics experiments. ^{125}I-FnHA quantification. A standard calibration curve was generated and used to determine the results for the loading and release kinetics experiments with correction for the half-life of ^{125}I ($R^2 = 0.995$).

Optimisation of ^{125}I-Fn coating of HA. The optimal time for loading of Fn onto HA discs was one hour (Fig. 1). Significant increases were seen in the amount of Fn remaining on the discs between all time-points up to one hour (all $p < 0.001$), but there was no significant difference between one and two hours ($p = 0.691$). The data show that there was no significant increase in the amount of protein retained on the discs after incubation for one hour.

After one hour (optimal incubation duration as shown above) the median maximum amount of Fn bound was 255.26 ng (95% CI 253.74 to 264.26) from an initial load of 1000 ng in 50 µl.

As the quantity of ^{125}I-Fn added increased (from 100 ng to 250 ng, and 500 ng to 1000 ng), a significantly higher quantity of ^{125}I-Fn remained on the discs (all $p < 0.001$); 50 µl droplets containing 1000 ng and 1500 ng did not produce proportionally more coupled protein ($p = 0.085$) (Fig. 2).

The optimal loading concentration and incubation time of 1000 ng in 50 µl for one hour was used as above to determine the optimum durability. A significant decrease from a median of 249.91 ng (95% CI 239.79 to 254.39) to 137.93 ng (95% CI 135.89 to 142.72 ng) of Fn coupled to HA was seen within the first hour of incubation in FCS ($p < 0.001$). There was no further decrease between one and four hours ($p = 0.233$), or between four and eight hours ($p = 0.1$); however, the amount decreased significantly to one-fifth of its initial optimal loading concentration (median 49.99 ng (95% CI 43.71 to 51.33)) by 24 hours ($p < 0.0001$) (Fig. 3). These figures are equivalent to 3.2 ng mm^{-2}, 1.8 ng mm^{-2} and 0.6 ng mm^{-2} of Fn on HA at zero, one to eight, and 24 hours, respectively.

Dermal fibroblast attachment experiments. For characterisation of the discs, the median R_a, R_z and S_m values for Pol

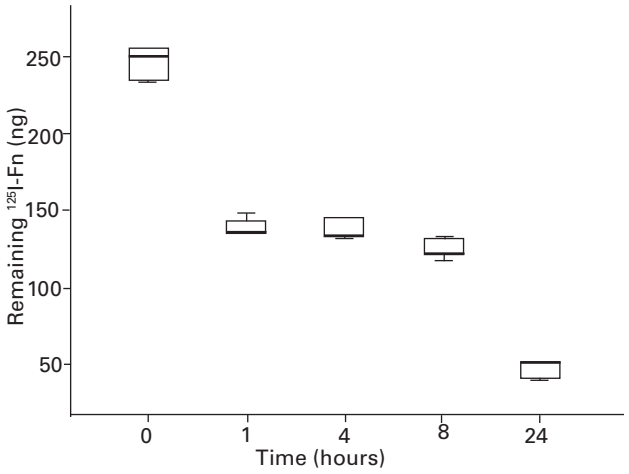

Fig. 3

Box plot showing of the amount of ^{125}I-Fn (in ng) remaining on HA surfaces with increasing incubation time (hours) after an initial loading of 1000 ng of ^{125}I-Fn in FCS. The boxes denote the median and interquartile range, and the whiskers denote the full range.

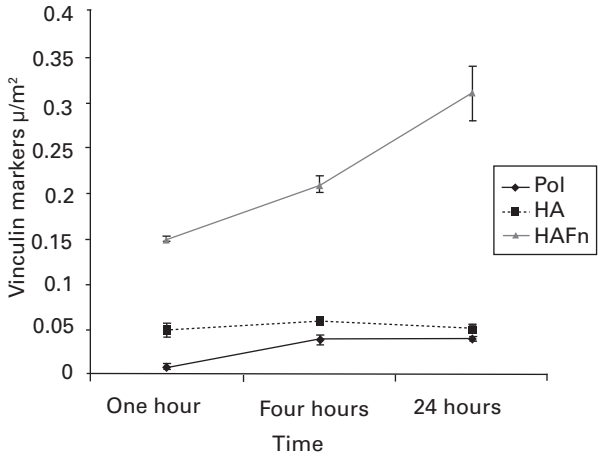

Fig. 4

Graph showing the median (with standard error bars) number of vinculin markers per unit cell area (count per µm^2) for the polished (Pol), hydroxyapatite (HA) and HA-fibronectin (HAFn) substrates at one, four and 24 hours.

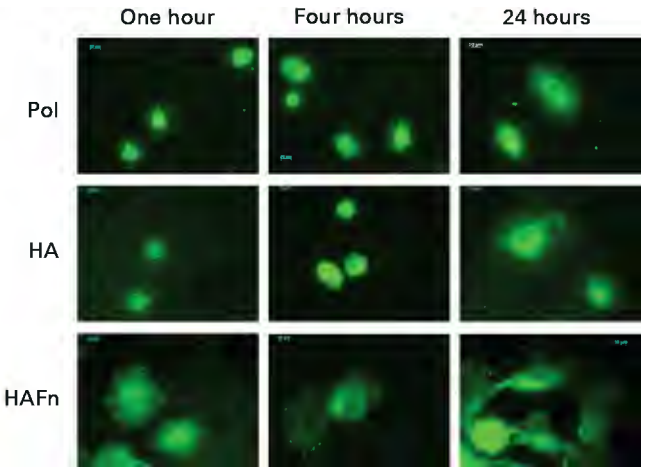

Fig. 5

Fluorescence microscopy images showing the appearance of fibroblasts on the polished (Pol), hydroxyapatite (HA) and HA-fibronectin (HAFn) substrates at one, four and 24 hours.

were 0.030 µm (95% CI 0.011 to 0.048), 0.120 µm (95% CI 0.100 to 0.148) and 20.630 µm (95% CI 9.804 to 32.701), respectively. The corresponding median values for HA were 0.039 µm (95% CI 0.121 to 0.052), 0.131 µm (95% CI 0.107 to 0.159) and 22.005 µm (95% CI 10.020 to 34.653). No statistically significant differences were observed between Pol and HA discs ($p = 0.650$, $p = 0.631$ and $p = 0.262$ for R_a, R_z and S_m, respectively).

Fibroblast focal adhesion and cell area quantification. Number of vinculin markers per cell. The number of vinculin markers per cell was significantly greater on HAFn than on the HA and Pol controls at all time-points (HAFn *vs* HA: $p = 0.003$, 0.004 and 0.004; HAFn *vs* Pol: $p = 0.003$, 0.004 and 0.004; at one, four and 24 hours, respectively). A 15-, 19- and 12-fold increase was seen with HAFn compared with HA alone at one, four and 24 hours, respectively. After one hour the number of vinculin markers per cell was significantly greater with HA than with Pol ($p = 0.006$), but by four and 24 hours the opposite was seen ($p = 0.025$ and 0.004, respectively).

Cell area. At one and four hours the cell area increased in the order HA < Pol < HAFn. The median cell area on HAFn was significantly greater than those on both HA and Pol controls (HAFn *vs* HA: $p = 0.003$ and 0.004; HAFn *vs* Pol: $p = 0.003$ and 0.004; at one and four hours, respectively). At 24 hours the cell areas on both HAFn and Pol were significantly greater than on HA ($p = 0.01$ and 0.004); there was no significant difference between them ($p = 0.631$). Cell area was observed to be 5-, 5.5- and two-fold greater on HAFn than on HA at one, four and 24 hours, respectively.

The attachment was measured by the number of vinculin markers per unit cell area. At one hour attachment increased significantly between Pol and HA ($p = 0.004$) and between HA and HAFn ($p = 0.003$) with a 14- and a threefold increase, respectively (Fig. 4).

A similar pattern was seen at four and 24 hours (Fig. 4); however, no significant difference was seen between Pol and HA ($p = 0.055$ and 0.150). Attachment was significantly greater on HAFn than on HA at four and 24 hours ($p = 0.004$): four- and sevenfold increases were seen.

On Pol substrates attachment increased significantly between one and four hours ($p = 0.004$), after which no significant difference was seen ($p = 0.199$).

Attachment of cells on HA was not significantly different between one and four ($p = 0.262$) or four and 24 hours ($p = 0.055$); however, on HAFn attachment increased significantly between both time points ($p = 0.038$ and 0.004, respectively) (Fig. 4).

Fluorescent microscopy. Figure 5 shows vinculin staining in cells on Pol, HA and HAFn at one, four and 24 hours. The

images show increases in cell area and vinculin markers on HAFn substrates at all times compared to HA and Pol controls. The attachment of cells, measured by the number of vinculin markers per unit cell area, on HAFn at one hour was 3.4 and 4.2 times greater than with HA and Pol at 24 hours (Fig. 5).

Discussion

In this study we have shown that HA can be functionalised by adsorption of Fn, and that optimising this procedure increases the attachment of dermal fibroblasts as measured by the quantification of vinculin markers per unit area of the cell.

Focal adhesions are critical in the regulation of cell attachment,[21,22] and quantification of the number of vinculin markers per unit cell area gives an accurate indication of the biophysical strength of cell attachment.[14]

Previous studies have shown that protein augmentation can increase the attachment of cells *in vitro*,[8,23-25] and attempts to create durable coatings by silanisation have shown promising results.[26] Silanisation techniques create –CHO bonds for protein binding, but are laboratory based and subject to considerable variability. Protein absorption may be a more consistent technique and, unlike silanisation, could be performed at the time of surgery for ITAP. Our findings show that after one hour of adsorption with an initial coating concentration of 13 ng mm^{-2} (1000 ng per 10 mm diameter disc), HA substrates are optimally loaded with 3.2 ng mm^{-2}, which significantly increases dermal fibroblast attachment *in vitro*. Given the duration of an ITAP surgical procedure, clinical implementation of our adsorption technique would be feasible.

In 2010, Gordon et al[26] showed that keratinocyte attachment could be increased by a coating of 6 to 7 ng mm^{-2} of silanised laminin-5. Our current findings agree with this, and show that between 3.2 and 0.6 ng mm^{-2} of Fn have a significant positive effect on fibroblast attachment. The maximum amount of Fn that could be adsorbed was 3.2 ng/mm^2, although we accept that this may not give a maximal increase in the attachment strength of the dermal fibroblasts. Moreover, it may not result in an equivalent increase in attachment *in vivo*, and further studies are needed to investigate this. We noted a decrease in adsorbed Fn on HA, only one-fifth of the initial load remaining by 24 hours. This shows that the stability of the coating is not as robust as that achieved with silanisation.[26] Despite this, a sevenfold increase in fibroblast attachment on Fn-functionalised HA was seen at 24 hours. Further investigations are necessary to determine whether this is directly due to the Fn coating or whether the initial coating influences the deposition rate and composition of the ECM, which in turn upregulates attachment.

In a study assessing the influence of the competitive preadsorption of human serum albumin and Fn on osteoblast adhesion and morphology, Sousa et al[27] concluded that the tissue response to implants is dependent on the initial attachment of cells to the substrate, and that this is directly related to the ability of cells to interact with the protein layer absorbed on the implant surface. In 2008, Laflamme and Rouabhia[28] showed that BMP-2 and -7 coatings promote osteoblast attachment to collagen scaffolds, and postulated that this was due to the substrate mimicking the *in vivo* physiological conditions of the ECM more precisely than uncoated controls. We suggest that Fn-pre-adsorbed HA resembles the adhesion protein component of the fibroblasts' native ECM more closely, enabling them to become attached more quickly and more efficiently than uncoated controls.

In conclusion, we propose that Fn-coated HA implants may improve dermal tissue attachment to an ITAP. An adsorption technique that applies Fn to HA-coated implants at the time of surgery may be enough to achieve this without the need for prolonged preparation, which might limit the application of these coatings. Further work is under way to determine whether increased concentrations of Fn result in further upregulation of dermal fibroblast attachment, and whether these coatings elicit a similar effect on dermal tissue attachment around an ITAP *in vivo*.

Supplementary material

Box plots showing the number of vinculin markers per cell and cell area for the polished (Pol), hydroxyapatite (HA) and HA-fibronectin (HAFn) substrates at a) one hour, b) four hours and c) 24 hours are available with the electronic version of this article on our website www.jbjs.boneandjoint.org.uk

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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