Defining the properties of an array of -NH_2 modified substrates for the induction of a mature osteoblast/osteocyte phenotype from a primary human osteoblast population using controlled nanotopography and surface chemistry

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Abstract

The accelerated integration of a joint replacement and the acceleration of fracture healing, particularly for complicated non-union fractures, should increase patient welfare and increase the efficiency of the healthcare provider. Currently, autologous bone graft is the gold standard method for the treatment of complicated non-union fractures, but this is not always possible. A proactive highly inductive so called smart material approach is pertinent in these cases; in this research altering surface chemistry of a previously approved material with desirable bulk material properties has been investigated to an approach that could be both economical and effective. The objective was to apply stable synthetic chemical coatings that can guide cells down and through the osteogenic lineage that is required to create a mineralised piece of tissue that could be fabricated in a cost effective manner to induce and accelerate bone healing.

Primary human osteoblast–like cells were cultured in vitro for 7, 14 and 28 days on a range, chain length 3-11, of amine terminated silane-modified glass surfaces, with controlled nanotopograpical profiles, to determine how changes in surface chemistry and nanotopography controlled osteoblast function . The material was characterised using atomic force microscopy (AFM), scanning electron microscopy (SEM), water contact angle (WCA) and a novel ninhydrin assay. . The cells were analysed using RT-PCR, Von Kossa tincutural staining for mineralisation, and visualised using both transmitted white light and electron microscopy. Bone-like nodules were only formed on the short chain (chain length 3 and 4) amines after 7 days and were quantified using microscopy. This, along with the up-regulation of sclerostin demonstrated that a specific combination of material factors resulted in a more mature osteoblast phenotype. The cellular response evidenced in this paper reported more rapid nodule formation than has previously been reported[1], and this occurred in the absence of exogenous factors in the media. This demonstrated the potential of the coating to improve the integration of bone biomaterials, or create a smart biomaterial that accelerates the bone regeneration process.

Introduction

10-15% of recorded bone fractures do not heal spontaneously within the first 6-8 week period and are then classed as non-union bone fractures. Non-union bone fractures are associated with an extensive fracture site, rendering the normal bone healing pathway inadequate [2]. These fractures present a significant challenge for the orthopaedic community and represent an area of modern health care where regenerative medicine strategies could have an immediate short term significant impact. The best current treatment is autologous bone graft, which is very successful when sufficient healthy bone is available. The major limitation therein is the availability of donor bone and the associated implications of bone harvesting and patient recovery [3][4]. One potential solution is the storage of bone for future use, a technique that has a good prognosis when treating chronic diseases [5] but the effectiveness of this is limited for treatment of acute trauma injuries. Joint replacement is also a significant area where increased osteoinduction and osteoconduction by biomaterials would be welcomed.

There is a significant and urgent need to develop synthetic materials that are osteoinductive and osteoconductive, that can be directly implanted into the site of a non-union fracture or joint replacement site, to control the response of mesenchymal stem cells (MSC) and osteoblasts. There is extensive research identifying selected surface/material properties that can be used to induce osteogenic differentiation of mesenchymal stem cells [6–8]**.** Data derived from these studies has demonstrated that MSCs can respond to stimuli at the sub-micron scale and are sensitive to changes in chemistry, topography and stiffness. In parallel there are studies that also report the beneficial effect of introducing an amine (-NH_2 or –NH_3) group to the surface of a substrate to improve the osteoconductive properties of a substrate[9][10].

The methods for introducing and enriching the substrates with the amine functionality have varied in reports and the material characterisation has not fully considered the combinatorial effects of

surface chemistry and nanotopography on the cell response. In addition the published reports have also not considered how changing the parameters of the amine enrichment protocol could affect the deposition of the amine group and the associated change in topographical profiles (a direct effect of introducing a chemical group to a surface). Therefore the correct combination of surface chemistry and nanotopography required to enhance osteoconduction i.e. the response of osteoblasts to a surface has not been fully investigated. As the process of natural bone healing differs when autologous bone is introduced (the correct matrix containing osteoblasts) [11], it is reasonable to assume that the material properties required for osteoinduction maybe different to osteoconduction. Regardless of the outcome it is a paradigm that must be investigated.

We have previously demonstrated that silane modification is a technique that can be utilised to introduce a chemical group to a surface i.e. $–NH_2$, on an array of base substrates/clinically relevant degradable polymers [12]. Changing the chain length of silanes used to produce an $–NH_2$ enriched monolayer is an effective tool for controlling the surface chemistry and associated nanotopography, controlling initial integrin binding, focal adhesion formation and subsequent mechano-transduction events. Silane modification has the potential to mimic the unique properties of bone extra cellular matrix by the addition of chemical and nanotopographical surface features that are comparable to ECM. Optimisation of these parameters would result in a surface that enhances the activity of osteoblasts and the production of substrate that could replace the need for autologous bone grafting. These novel materials would be chemically stable and require no manipulation with biological agents prior to implantation, therefore potentially providing cost effective directly implantable osteoconductive materials that can stocked and stored for future use (of the shelf material). The surface modifications could be introduced to particulate or scaffold type matrices, as well as solid substrates (metallic implants) as required; highlighting the significant impact the versatility of this approach could have and the need to identify specific material/surface parameters that can be used to control osteoblast responses as well as other tissue.

This research has investigated a range of $–NH_2$ presenting silane modified layers. The chain length of the silanes was used as a variable to control chemical group deposition, via the formation of

a cohesive monolayer and nanotopography. -NH_2 groups on the surfaces were quantified using ninhydrin assays and the hydrophobicity of the surfaces was quantified using water contact angle analysis. Topographical profiles were analysed using AFM. Primary human osteoblasts were cultured in contact with the tests surfaces and adhesion, morphology, phenotype and the production of calcified nodules/matrix was examined using light and electron microscopy and real time polymerase chain reaction (RT-PCR), identifying markers of osteoblast maturation and osteocytes. [13, 14] All cultures were conducted in basal conditions.

Methods and materials

Preparation and modification of borosilicate glass.

12mm diameter glass coverslips (SLS, UK) were cleaned using a 0.5M sodium hydroxide (Sigma, UK) for 30 minutes (Fisher, UK), 3 changes of distilled water, and 1M nitric acid (Sigma, UK) for 30 then 3 changes of distilled water (all changes in an ultrasonic bath), and dried at 50^{o}C. Glass was submerged in 0.1M solutions of the following; (3-Aminopropyl)triethoxysilane (CL3) (Sigma), 4-(triethoxyslyl)butan-1-amine (CL4) (fluorochem), 3-(2-Aminoethylamino) ropyldimethoxymethylsilane (CL6)(Sigma), N-(6-Aminohexyl) amnomethyltriethoxysilane (CL7)(Fluorochem) and 11-Aminoundecyltriethoxysilane (CL11)(Fluorochem), for 30 minutes at room temperature then washed with isopropyl alcohol and distilled water for 5 minutes.

Atomic force microscopy (AFM)

Unmodified glass controls and modified samples were attached to glass microscope slides using double sided adhesive tape and examined using an AFM microscope (Zeiss). 4 areas from 3 samples of each modification were scanned in tapping mode over a 500nm scan area. Images were analysed using Asylum Research Argile Lite Programme.**Water contact angle measurements**

These were recorded using a Camtel Dynamic Contact Angle machine (Camtel Ltd. UK). Substrates that had been modified on both sides were immersed into water and the dynamic contact

angle measurements recorded. A total of 6 repeats were recorded for each modification, results presented as average +/- SD and data analysed using ANOVA to test for statistical significance.

Ninhydrin assay on films and glass

The concentration of the $–NH_2$ groups were measured using a novel ninhydrin assay, to visualise and quantify the concentration of amine groups on the surface. A clean glass coverslip was submerged in a 0.1M silane solution for 30 minutes to apply the –NH2 coating as detailed above. After this process 1mL of the ninhydrin solution (0.35g of ninhydrin (Sigma, UK) was dissolved in 100mL of ethanol) was added to the material and its coating solution then heated to 90^{o}C in an oven for 5 minutes. The solution was removed from the coverslips and diluted 1:3 with ethanol. The reagent changed colour in presence of amine from yellow to purple. The coverslips were imaged and photographed using transmitted light microscopy once the solution had been removed to show the distribution of the amine groups across the surfaces. Amine group deposition was visualised as purple staining. The ninhydrin/coating solution that was removed was run alongside a standard curve of known silane concentrations (ranging from 20-120μM this was determined through optimisation of the assay) to measure the concentration remaining in the solution. It was possible to determine the concentration of the $–NH_2$ groups that had been deposited on the surface of the material by measuring the concentration of the coating solution and calculating the loss of concentration.

Isolation of primary human osteoblast-like cells

Human osteoblast-like cells were cultured from explants of human bone taken from ethically approved osteoarthritis surgery were washed with a solution of PBS with streptomycin and penicillin (Sigma, UK). They were then cut into 1-2mm diameter pieces, and 6-8 of the bone pieces were placed in a 9cm diameter disposable petri dish, cultured with 10ml of DMEM media plus 5% foetal bovine serum (Sigma, UK). Cultures were incubated for 6-8 weeks, until cells formed a confluent monolayer.[15][16]

Primary human osteoblast like cells on silane modified glass

$5x10^4$ (in 100μL media) of well characterised primary human osteoblasts were seeded onto the glass surfaces and incubated at 37ºC for 1 hour in a 24 well plate. Following this 2ml of DMEM media with 5% foetal bovine serum (Sigma, UK) was added to each well and incubated at 37ºC, 5% CO_2 for 7, 14 and 28 days. 1 mL of media was removed and 1mL of fresh media was added at 14 days. At the appropriate time points samples were removed from culture and analysed using von Kossa (calcified extra cellular matrix), scanning electron microscopy (SEM) and real- time polymerase chain reaction (RT-PCR).

Von Kossa staining of coverslips with primary human osteoblast like cells

At the appropriate time point, cells were removed from culture and washed with PBS.. Cells were fixed using a 2% formaldehyde 4% sucrose solution for 15 minutes at 37ºC and then washed with PBS and distilled water. Samples were transferred to 1% silver nitrite solution for 1 hour under UV light. Following this samples were washed and placed into a 2.5%(w/v) sodium thiosulphate solution for 15 minutes at room temperature, counterstained with Weigerts haematoxylin for 3 minutes, washed in tap water for 5 minutes, (Sigma, UK) then mounted on a microscope slide using aqueous mountant (Sigma, UK), and imaged using a Axio Vision microscope (Zeiss, Germany)this was repeated 3 times for each timepoint. Representative images are used in results section.

Calcified nodule count

Nodules were counted for each sample by setting light microscope to a low magnification (2.5 x objective) and observing all of the sample in one field of view. Nodule count was recorded for each sample (N=16).

Scanning electron microscopy (SEM)

Samples were removed from culture, washed with PBS and fixed using 2.5% (w/v) glutaraldehyde for 15 minutes at room temperature, washed with PBS, and submerged in 70% ethanol for 15 minutes, then 90% ethanol for 15 minutes followed by two changes of 100% ethanol for 15

minutes. Samples were dried using a critical point dryer (Prion, UK). Dry samples were fixed to SEM stubs (Agar Scientific, UK) using double sided carbon sticky tabs (Agar Scientific, UK). The samples were then coated with 20nm of chromium using a sputter coater (EMTECH, UK). The samples were observed under a Leo 1550 FESEM (Zeiss, UK). This was repeated 3 times for each timepoint. Representative images were used in the results section. Where nodule formation was found measurements were taken using the annotation function on the LEOSEM software (Zeiss UK).

Preparation of RNA using trizol

Samples were removed from culture and placed into clean 24 well tissue culture plates (SLS Ltd, UK) washed using sterile Dulbecco's PBS (Sigma, UK) to remove any non-adherent cells. 500µl of Trizol (Sigma, UK) was added to each well for 5 minutes. Trizol reagent was then removed from the coverslips and frozen until required at -80 ^{o}C.

100µl of Chloroform (BDH, UK) was added to defrosted samples and vortex mixed. Samples were spun at 18,000g for 5 minutes and the upper layer removed into a new tube. 300µl of isopropanol (Sigma, UK) was then added, and centrifuged at 18,000g for 15 minutes. The supernatant was removed, 500µl of 100% ethanol was added and centrifuged for 5 minutes(18,000g). The supernatant was removed and 200µl of 70% ethanol and spun for 2 minutes at 18,000g. Pellet was re-suspended 10µl of DNA/RNA-free ultra-pure water. (Sigma, UK) Generic DNA contamination was eliminated using commercially available DNAse kits (Invitrogen UK) according to the manufacturer instructions. First strand cDNA was synthesised using kit (Invitrogen, UK) following manufacturer instructions.

RT-PCR

Quantitative real-time polymerase chain reaction (RT-PCR) was conducted using primers for Osteopontin, Osteocalcin, Osteonectin, Collagen I, Collagen II, and CBFA-1 and sclerostin (figure 7), with all results normalised against the housekeeping gene β-Actin using ΔΔct equation. 20µl of

forward and 20µl of reverse primers were mixed with 160µl of DNA/RNA-free water. The following reagents were added in triplicate to the wells of a RT-PCR 96 well plate (BIO-RAD, UK): 2µl cDNA template, 7.5µl SyBR green (BIO-RAD, UK), 4.5µl DNA/RNA-free water, and 1µl diluted primer (as above). RT-PCR was conducted using i-cycler (BIO-RAD,UK), using previously defined optimum temperatures for each primer (data not included). Lack of contamination of samples was confirmed using standard melt curve analysis. For each sample and each gene a total of 6 repeats were carried out. Statistical analysis was carried out using ANNOVA.

Results

Atomic force microscopy (AFM)

AFM data demonstrated different topographical profiles were present when different chain length were used. The AFM images of CL3 and CL4 (figure 1a and 1b) were similar in height and feature size – both having maximum feature heights of under 10nm. The CL7 modification (figure 1d) showed the formation of clumps of matter which form ridges. This pattern was also seen on the CL6 (figure 1c) surface.

Water contact angle measurements

When the variance of mean was examined using ANOVA ($p=\geq 0.05$) CL3, CL4 and CL11 showed a significantly different water contact angle when compared to the control, and to CL6 and CL7. CL6 and CL7 did not differ significantly from the control (figure 2a).

Ninhydrin on films and glass

The ninhydrin assay for amine concentration (figure 2b) demonstrated that its concentration on CL7 was significantly less than any of the other modifications when analysed using ANOVA ($p=\geq 0.05$).

Von Kossa staining osteoblast like cells on modified materials

Qualitative evaluation of the cells cultured in contact with the surfaces and stained with von-Kossa for the production of a calcified extra cellular matrix demonstrated that all surfaces supported osteoblast like cell adhesion at all time points tested (figure 3). In addition to supporting viable cell adhesion, cells cultured on CL3 and and CL4 $–NH_2$ modified substrates (figure 3b and c) produced calcified nodules at various points on the surfaces by day 7, this response was not observed on the control or other $–NH_2$ modified surfaces. Cells cultured on CL3 and CL4 surfaces maintained their ability to continuously produce calcified nodules throughout the 28 day test period.

Nodule count using light microscopy

The size and number of cell clusters formed on the CL3 and CL4 substrates where significantly higher than the cell clusters formed on any of the other modifications. The numbers of nodules on each sample were counted by light microscopy (figure 4a).

Scanning electron microscopy and nodule measurement (SEM)

Cellular interaction with the control and modified substrates was further evaluated using SEM analysis (figure 5). In line with previous observations representative SEM micrographs showed that all test substrates supported cell monolayer attachment and the production of an extra cellular matrix. Once again there was evidence of the formation of cell clusters forming on CL3 and CL4 substrates (figures 5b and c). SEM micrographs clearly show that the cell clusters are encased in a cohesive extra cellular matrix, supporting the definition of calcified cell nodules. This phenomena was not observed on any other test or control substrate at any time point. The size of nodules were measured using the measuring facility on the SEM software (Zeiss SEM user interface)(figure 4b). The size of the nodules increased significantly over 14 day period on the CL4 modification, then reduced significantly at 28 days.

Real time polymerase chain reaction (RT-PCR)

The data is presented as an up-regulation in activity per cell compared to the glass control. Cells cultured on CL3 surfaces did not show a sustained up regulation of osteogenic markers, compared to the glass control at all time points. In contrast cells cultured on CL4 modified substrates demonstrated an up regaultion of osteopontin (day 14), osteocalcin (day 7), osteonectin (day 7) and collagen I (day 28). Cells cultured on both CL3 and CL4 substrates demonstrated a sustained up-regulation of sclerostin from day 14 (figure 6e). This was statistically significant on both the CL3 and CL4 surface at day 14. Cells cultured on CL6, CL7 and CL11 modified substrates did not demonstrate a sustained up-regulation of scleorostin at the same magnitude as CL3 and CL4 substrates. Cells cultured on CL6, CL7 and CL11 substrates again did not demonstrate a sustained up-regulation of osteogenic markers throughout the test period, apart from the expression of osteocalcin on CL11 substrates at day 28 which was significantly enhanced. This finding was in line with the enhanced extra cellular matrix observed in figure 5f.

Discussion

Previous work has evaluated the potential of silane modified substrates, specifically $–NH_2$ modified substrates as a tool for the osteoinduction of mesenchymal stem cells. The data from these studies has demonstrated that silane modification can be used to induce changes to material surface properties at the sub-micron scale, combining the optimal parameters of surface chemistry and nanotopography, which in turn can be used to control initial cell adhesion and ultimate cell response[12] [6] [17][18][19][20]. To date the effect of these substrates on differentiated cell types i.e. osteoblasts has not been established nor has the optimal combination of material chemistry and nanotopography required to enhance osteoblast activity been established. [21][22][1]. To optimise the potential of $–NH_2$ silane modified surfaces in both in vitro osteoblast culture, and as coatings for orthopaedic devices, the effect of $–NH_2$ enriched surfaces with controlled nanotopography must be understood.

The material properties of the surfaces were changed significantly when the silanes were applied, this was clearly shown by changes in water contact angle and the concentration of amine

associated with each of the test substrates. The variables were reproducible on all surfaces, and dependent on the chain length used to produce the monolayer. Results demonstrated that the amine concentration on CL7 was significantly less than any of the other modifications. When taken in conjunction with data derived from the Ninhydrin assays, there was a correlation between the significantly lower concentration of amine on the CL7 surface and the significantly reduced WCA. These results suggest that the concentration of amine is responsible for the change seen in the water contact angle. The reduction in amine concentration and associated WCA could be attributed to the fact that CL7 amine chains clustered during the silanisation process did not form a complete self-assembled monolayer (SAM). This hypothesis was further strengthened by the presence of clumps of matter which form relatively (from a nano-topography standpoint) large ridges (figure 1d) on the AFM micrographs. The presence of clumps of material would result in a reduction in stability of the surface, which was less likely to withstand the vigorous washing procedure associated with the surface characterisation techniques[23]. These results support that the CL7 modification process should not be used to produce surfaces that are intended for cell culture, or direct implantation into the body, as the stability of the coating cannot be verified. This would result in a non-uniform cellular response.

The AFM highlighted the differences in nanotopography (figure 1). AFM showed that there were differences between the silanes and supported the idea that the CL3 (1a) and CL4 (1b) offer a consistent surface roughness.

Taken together, the three characterization techniques (AFM, Ninhydrin and WCA) demonstrated that the silanes used in this study successfully altered the glass substrate. They created different surface topographies when examined on the nanoscale, in addition to the change in surface chemistry. There was an increased concentration of amine groups after modification. Amine groups have been demonstrated in previous studies to show osteogenic capacity[8, 24]. Previous research has focused around the influence of surface nanotopography on stem cells and it has been demonstrated that there is an optimum surface topography for osteogenic differentiation [25]. Key points from the

studies that were successful in creating an osteogenic topography were they created a surface that had an optimal surface roughness, and it was not ordered.

The Von Kossa stain on the osteoblasts cultured on the silane modified surfaces revealed several significant effects. There was a response on the CL3 and CL4 treated surfaces which lead to the formation of mineralised nodules by 7 days. This response was maintained throughout the culture period of 28 days and was limited to the CL3 and CL4 modifications. A statistically insignificant number of nodules were seen on the other modifications which supported cell expansion throughout the culture period, but with no observed change in phenotype during this time. The nodule number was counted visually, and the resulting data displayed in figure 4a. The nodule number was significantly reduced at 28 days on both CL3 and CL4, when compared to 14 day figures for the same modification analysed by T-test, the results showed significant differences to the 95% confidence interval. There was a significant increase between CL4 at 7 days and CL4 at 14 days (to a 95% confidence interval), whereas there was a plateau between 7 and 14 days on CL3. The nodule formation is an important process in the life cycle of the osteoblast – it is one of the main defining factors that can distinguish between osteoblastic activity and osteocytic activity. The presence of nodules is a clear indicator of the continued differentiation of these cells.

The SEM images (fig 5) clearly demonstrated nodule formation. The nodules on CL3 (fig 5b) and CL4 (fig 5c) appeared to be covered in extracellular matrix, which matured with time. This mineralisation was also demonstrated by the positive von Kossa staining from early time points in the culture period. Extracellular matrix was also produced in abundance on the CL11 surface, but in the absence of nodule formation to any significant degree.

The primary human osteoblast-like cell model utilised represented an advanced stage in the osteogenic pathway. Examining how these cells interact with the modified surfaces was conducted to demonstrate how mature terminally differentiated cells reacted to the surface modifications, giving an insight to what the longer term effects may be. There was an interesting response observed at the first time point; Von Kossa staining (fig 3) showed mineralized nodules formation on the CL3 and CL4

surfaces. This reaction was not seen on the other surfaces, where the cells remained in monolayer throughout the 28 day period. The nodule formation on CL3 and CL4 could be explained by the progression of the osteogenic differentiation of the cells. Primary human osteoblasts have been shown to form nodules[22] when in 3D culture on bioactive glass, but only in the presence of exogenous growth factors. This stimulus has come purely from the material.

The formation of nodules on the CL3 and CL4 surfaces was confirmed by SEM, and Von Kossa staining. As the cells used in this model were already capable of producing ECM, all of the samples including the untreated glass control stained positive for mineralization (Von Kossa) at 7 days, however the cells on the untreated control were less densely mineralized by 28 days than the silane treated samples. The cells on CL6, CL7 and CL11 all retained the ability to produce matrix which became mineralized.

The formation of nodules supports the hypothesis that a population of the cells were starting to gain the characteristics of osteocytes. The embedding of the cells in a highly dense matrix is an important physical step along the pathway towards osteocytes, which have been shown to be important in the regulation of the osteogenic process

The size of the nodules (figure 4b) was shown to increase over a 14 day period and then significantly decrease. This occurrence when combined with the statistically significant drop in the total number of nodules (figure 4a) at 28 days could be indicative of the nodules reaching a critical size and then detaching from the surface. Interestingly on CL3 and CL4 the expression of sclerostin was notable at 14 and 28 days. Sclerostin is a Wnt antagonist and is specifically a marker of osteocytic activity[14]. This along with the reduction of normal osteoblastic markers after 7 days is indicative of the further differentiation of the cells along the osteogenic pathway. All the results are consistent with the hypothesis that the materials can influence osteoblast-like cell fate, and cause them to express osteocyte specific markers. Scelorostin is only expressed on the materials (CL3 and CL4) which have the nodule formation, indicating that the sclerostin is specific to cells embedded in matrix, which is consistent with current opinion that sclerostin is a marker specific to embedded osteocytes.

The differences observed between the chain lengths and their osteogenic effects can be attributed to, the topography that is induced at the nanoscale, and the mimicry of the correct ECM by the chemical modification. In the instance of the CL3 and CL4 nodule formation, it is possible that the amine rich surfaces and the topography instigated the formation of nodules, and the expression of osteocytic markers. This work leads to the conclusion that it is possible to use inexpensive surface chemistry and topography modifications to mimic the role of ECM in the osteogenic differentiation pathway. This ultimately could lead to a synthetic, ecomomic off the shelf material suitable for bone regeneration applications in currently difficult to treat medical conditions.

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Figure legends:

Fig 1 AFM microscopy of different chain length silanes. **Fig 1a** CL3 modification, **Fig 1b** CL4, **Fig 1c** CL6, **Fig 1d** CL7, **Fig 1e** CL11 and **Fig 1f** unmodified glass. Silane modifications were added to glass substrate and AFM microscopy was performed in tapping mode. AFM microscopy shows the topographical differences seen on the nanoscale

Fig 2a Dynamic Water Contact Angle Chart showing dynamic water contact angle of a control glass and glass modified with CL3, CL4, CL6, CL7, and CL11. Stars indicate statistical significance ($p>0.05\%$) **Fig 2b** Ninhydrin assay for amine concentration Chart showing concentration of amine groups on surfaces. This demonstrated that the concentration on CL7 was significantly less than any of the other modifications when analysed using ANOVA ($p=\geq 0.05$)

Fig 3 Osteoblast-like cells cultured on silane modified glass for 7 days. Osteoblast like cells were cultured on the silane modified glass (and an untreated control) for 7 days, then stained with Von Kossa's stain for mineralisation **Fig 3a** untreated glass control, **Fig 3b** CL3, **Fig 3c** CL4, **Fig 3d** CL6, **Fig 3e** CL7 and **Fig 3f** CL11

Fig 4a Quantity of nodules formed on the modified surfaces. The nodules were counted using a light microscope. (N=16) Series 1,2 and 3 correspond to 7, 14 and 28 days, results show average and error bars show standards deviation from the mean. **Fig 4b** Size of nodules on the modified surfaces. Nodules on surfaces treated with CL3 and CL4 were measured after 7,14 and 28 days results show average and error bars show standards deviation from the mean

Fig 5 SEM micrographs of Osteoblast-like cells cultured on silane modified glass after 28 days incubation. Osteoblast –like cells were isolated from human trabecular bone and seeded onto the silane modified surfaces. **Fig 5a** untreated glass, **Fig 5b** CL3, **Fig 5c** CL4, **Fig 5d** CL6, **Fig 5e** CL7 and **Fig 5f** CL11. White arrows indicate nodules

Fig 6 Expression of osteopontin(**Fig 6a**), osteocalcin(**Fig 6b**), osteonectin(**Fig 6c**), collagen I(**Fig 6d**) and sclerostin(**Fig 6e**) in human osteoblast like cells after 7, 14 and 28 day incubation with silane modified glass. Osteoblast like cells were isolated from human trabecular bone and processed for rtPCR. Expression of osteopontin was measured and normalised to expression of B-Actin and

unmodified scaffold. Data shown is average expression and standard deviation from mean. *=p<0.10, **=p<0.05, ***=p=<0.01

Statement of Authors:

Sandra Fawcett wrote first draft and designed and conducted experiments, with the practical, experimental and academic assistance of Judith Curran, Rui Chen, Peter Wilson, Lakshminarayan Ranganath, Jane P Dillon, James A Gallagher[1] and John A. Hunt[1]

Informed consent was taken from all patient donors.