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Original Article

Sintered nanoporous biosilica diatom frustules as high efficiency cell-growth and bone-mineralisation platforms

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In this paper, we disclose a novel biomaterial with a considerable capacity for cell growth and bone mineralisation. The biomaterial is manufactured by sintering diatom (*Cyclotella sp.*) frustules under two different moulding pressures at temperatures of 1100°C over varied times (5-72 hours). The *in vitro* cell biocompatibility of the biomaterial was initially assessed using human kidney HEK cells and MDA-MB-231 breast cancer cells. These cells attached to the biomaterial and were in fact observed to grow preferentially on the diatomaceous biomaterial in larger densities than on commercial cell culture plates over longer time-periods (> 15 days). As part of a bone-regeneration proof-of-principle study, cell biocompatibility of the material was also tested *in vitro* using pre-osteoblast MC3T3-E1 cells over 21 days and osteoblast activities were measured by staining with von-Kossa stain for mineral deposits. Cells had attached to the biomaterial on day 2 and showed positive staining for mineral deposits after the 21-day period. The material was also noted to be autoclavable and reusable without any adverse effect observed on subsequent cell cultures.

Keywords:

Biosilica, Cell-Growth, Cell-Culture, Biomaterial, Bone-Graft

1. Statement of Significance

The work is significant as it demonstrates how a ubiquitously available inexpensive material (diatomaceous earth) can be manufactured to form 3D cell-culture platforms, without any loss in the nano-architectures of each diatom cell wall, which benefits cell-adhesion and colony formation. Perhaps more significantly, we show that cells preferentially adhere to our diatomaceous material, rather than to platforms manufactured specifically for cell-culturing. Since our material can also be subtractively manufactured into 3D shapes, we envisage it has potential significance as a 3D cell-culture platform, bioreactor/fermentation technologies, or as bone-grafting materials; a proof-of-principle for which is provided herein.

2. Introduction

Advances in the development of novel biomaterials are driven by factors such as material availability, biocompatibility, economic practicality, ease-of-manufacturing, and end-use. Biomaterials are engineered substances from either natural or synthetic sources, that interact with living systems to control the course of therapeutic or diagnostic procedures. There is a plethora of biomaterials today that have been developed to be biocompatible and non-toxic to cells. However, the lack of cell-toxicity does not always correlate with cell-growth and cells may only thrive on certain substrates. This is usually very evident when observing attached cell structures and morphologies, which become less distinct on lower compatibility biomaterials, as do the cell proliferation rates. Manufacturing biomaterials with heightened properties of cell-proliferation is not a trivial task. Improved cell-interaction is usually

achieved through physical and/or chemical modifications to the material. Chemical modifications include the two major families of integrins and proteoglycans, which improve cell adhesion to biomaterials through focal adhesions with connective tissues [1]. Biopolymer templates such as gelatins [2], biomimetic extracellular matrix proteins [3], hyaluronans [4], polylactic acids [5, 6], and alginates [7, 8] are also shown to improve cell-proliferation once grafted to the surface of a host material.

Since cell adhesion and proliferation can also be improved by physically modifying a material surface, chemical grafting is often coupled to changes made to the topography [9]. A noteworthy topographical refinement involves coupling the vertical alignment of TiO₂ nanotubes with a judicious spacing arrangement onto material surfaces. This modification to the material surface alone results in a 300-400% increase in the proliferation rate of osteoblasts [10]. The research reported by Oh and co-workers [10] highlights the importance of nanoscale surface engineering as a means to improving the health of adhering and proliferating cells. There are numerous methods that can be used to nanoengineer a cell-growth substrate surface including; structural self-assembly [11], anodising oxidised surfaces [12], laser-engineering [13] and patterned differential stiffness hydrogels [14].

Silica nanoparticulate coatings have shown some promise in inducing cell proliferation. Pelaez-Vargas and co-workers [15] for example, demonstrated that silica coatings on zirconia substrates supported osteoblastic cell adhesion and proliferation. The work of Porte-Durrieu et al [16] demonstrated cell adhesion onto silica surfaces could be facilitated by covalently functionalising the silica with the cell-adhesion peptide sequence ArgGlyAsp. Importantly, silica can be functionalised with relative ease as it has a high affinity to osteogenic growth peptide adsorption and is consequently able to promote osteoblastic growth both, as neat silica, and as a silica/apatite composite [17]. Silica/apatite composites have also been manufactured using diatomaceous earth (SA-DE) (mined deposits of biosilica exoskeletons (frustules) of microalgal diatoms) [18] and compared against both tissue-culture polystyrene platforms (TCPS) and silica/apatite using synthetically manufactured silica (SA-SM). Though the SA-DE showed marginal improvements in osteoblast growth over SA-SM, neither of these showed any improvement over TCPS. All platforms did however show the cells were growing with primarily a healthy polygonal morphology. A point of note on the research nevertheless, is that on observing the scanning electron microscope images, it is evident that through the manufacture of SA-DE as a composite, much of the nano-topographical detail that ordinarily exists on diatom surfaces is lost. Diatom frustules are ornamented with nanopores that create a bio-glass with an elaborate hierarchical solid state continuum. Since as discussed earlier, nano-architectures improve cell-adhesion and proliferation, it would seem beneficial to try and preserve such a structure. Diatom frustules are es-

entially, protective houses for single celled organisms and one could easily hypothesise thence, that the structures of diatom frustules benefit cell longevity, the lifespan of which can theoretically be indefinite [19]. Though diatom frustules have not been researched extensively in view of their cell grow-potential, there are a number of recent reports that show frustules to be highly promising materials upon which living cells can form healthy biointerfaces. *Thalassiosira weissflogii* frustules for example, show excellent excellent NHDF and Saos-2 cell-compatibility as bare clean frustules. When surface grafted with -SH the frustules actually enable improved cell-compatibility, though grafting the frustules with -NH₂ reduces cell-compatibility and the merging of these two grafting methods may be a route to controlling cell-growth patterns on biomaterials [20]. Doping *Thalassiosira weissflogii* frustules with Ca²⁺ ions has also shown itself to be promising for Saos-2 cell-compatibility [21] yielding almost double the number of cells as compared to untreated frustules, and close to triple the number of cells grown on glass. TEMPO oxidation of *Thalassiosira weissflogii* enables improved fibroblast and osteoblast compatibility [22], while levels of osteoactivity between frustules is increased by functionalising the surfaces with sodium alendronate, which increases the hydrophilicity of the material [23]. Importantly, osteoactivity is improved when using mesoporous surfaces such as diatom frustules instead of smooth surfaces [22], with biosilica valves reported as promoting osteoblast cell growth more effectively than biosilica rods, clarifying the relevance of nanotexturisation as a parameter for cell growth [24].

In this paper, we aim to sinter pure diatom frustule continua to create viable 3D cell growth platforms with an application in bone-grafting, and potentially other 3D cell-growth technologies. We hypothesise that pure biosilica as a continuum structure, with the preserved frustule architectures, will create a platform conducive to healthy cell growth as the cells will attach to a nanostructured material that originally existed as a material in continuous intimate contact with single celled organisms.

3. Methods

3.1. Manufacture of nanoporous biosilica biomaterials

Diatomaceous earth (Diature - Natural Feeds and Fertilisers Ltd (NFF)), comprised of *Cyclotella sp.* diatom frustules sourced from fresh water deposits, was used in the sintering process to form solid-state continua of fused diatom frustules (hereinafter: FDF). The physical and chemical properties of the diatomaceous earth are shown in Tables 1 and 2, respectively, and were provided by NFF. There is considerable inherent damage of the frustule material since they are several thousands of years old, and this naturally increases the surface area of the frustules shown in Table 1, and moreover accounts for many of the smaller PSD values reported in this table. A total of

112 samples were prepared in ceramic crucibles filled with powdered diatomaceous earth and subjected to moulding pressures under high heat (1100°C), with an aim of accelerating the rate of solid-state diffusion between the diatom frustules to form a continuum biomaterial of nanoporous biosilica. Table 3 provides the manufacturing rubric for this work. The final materials were cut into cuboids using a Proxxon 37172 Micro Band Saw MBS/E. Each block was weighed and dimensions were measured to calculate the densities of each manufacturing condition.

3.2. Scanning electron microscopy (SEM)

A Jeol JSM-6335-F scanning electron microscope (SEM) was used to characterise the sintered biosilica diatoms. The SEM was operated at 15kV and the samples were sputter coated with platinum.

3.3. Mechanical testing of sintered biosilica diatoms

Biomaterial blocks were subjected to compressive loading at a speed of 5 mm per minute using an Instron (8872) servo hydraulic test machine (1.5kN load cell capacity). Force outputs were converted to 1st Piola-Kirchhoff (engineering) stresses and crosshead displacement was used to determine the Lagrangian (engineering) strains. The crosshead was calibrated using a thick piece of steel to ensure all variations arising from crosshead movement were accounted for in the strain calculations.

3.4. Biocompatibility testing of the material with different cell types

Human embryonic kidney cells (HEK-293) stably transfected with the AmCyan-P2A-mCherry plasmid [25] (available in Addgene plasmid #45350) and MDA-MB-231 breast cancer cells were cultured in Dulbecco's Modified Eagle Medium/F-12 (DMEM/F12) (DMEM/F-12; Gibco, Cat#11320033) cell culture medium supplemented with 10% fetal calf serum (FCS), 50 U/ml of penicillin and 50 mcg/ml streptomycin (both - from Gibco, Cat#15140122). The cells were maintained at 37°C in humidified atmosphere with 5% CO₂. Cells were grown and maintained in culture flasks until trypsinised and re-plated on 6-well plates for the experiments at a density of 50-100,000 cells per well. Cell culture media was exchanged for fresh medium every two days. Visualisation of the cells was performed using an EVOS FL microscope (Thermo Fisher scientific). Viability was analysed by the use of viability dye Calcein-AM (Thermo Fisher Scientific). Pre-osteoblast MC3T3-E1 cells were cultured in alpha minimum essential medium (α -MEM) cell nutrient medium supplemented with 10% fetal bovine serum (FBS USA). This cell line was a gift by Dr Riku Kiviranta, Department of Medical Biochemistry and Genetics, University of Turku, Finland. Additional confocal images were taken using a Nikon Ti Eclipse Confocal Microscope equipped with Plan Apo VC 100 $\times$ 1.4 Oil Dic N2 objective, which uses a laser for fluorescence detection. Images were rendered in NisElements

AR software (Nikon). HEK-293T cells were stained with Alexa fluor 400 phalloidin (Invitrogen) and NucBlue (Invitrogen), or transfected with a plasmid expressing CIB1 protein fused with mitochondrial targeting signal and GFP using Turbofect reagent (ThermoScientific).

3.5. Differentiation of MC3T3 cells

MC3T3 cells, isolated from mouse calvarial at early stages of differentiation, were cultured in T75 cm flasks until 60% confluency. They were then trypsinised and counted using a haemocytometer, before being plated onto the material. 1 ml of MC3T3 pre-osteoblast cell suspension (75 $\times$ 10³ cells per well) was seeded onto the FDF in 6-well plate, and cultured for the next 48h in a tissue culture incubator (37°C and 5% CO₂) for the cells to grow to near-confluency. After 48 h the medium was replaced by 2 ml of osteogenic medium (α -MEM full medium conditioned with 10 μ M dexamethasone, 50 μ g of ascorbic acid, and 5 mM Na-beta-glycerophosphate). The medium was replaced by fresh osteogenic medium every 2 days for the next 21 days.

3.6. Mineralisation by von-Kossa assay

Fragments of FDF cultured with or without (control) pre-osteoblasts were stained as follows: the samples were washed 3 times with dH₂O, followed by addition of enough 2.5% nitrate solution (protected from light) to cover the FDFs, and incubated for 30 minutes. Samples were exposed to bright light to enhance the reaction for 30 minutes. The silver nitrate solution was then suctioned off and the FDFs were rinsed with dH₂O three times. The FDF was covered with dH₂O and washed to ensure all silver nitrate was removed. The FDF fragments were then imaged under a stereo microscope (AxioCam ICc3 Carl Zeiss Lumar V12).

3.7. Sterilisation of the diatomaceous material

The FDF material was washed with dH₂O/96% ethanol and then autoclaved using standard procedures.

4. Results

4.1. Characteristics and mechanical properties of FDF

All samples regardless of heating time and pressure were successfully diffusion bonded through a sintering process. Solid state diffusion between diatom frustules is, therefore, possible under the conditions reported herein. FDF can be easily manufactured into many shapes (in both 2D and 3D) with the mere application of heat and pressure, and they can easily be subjected to post-sintering subtractive manufacturing. Figure 1(a) shows the diatomaceous earth prior to sintering, and Figure 1(b) shows post-sintered, subtractive-manufactured 3D geometrical primitives of FDF.

The scanning electron micrograph images, Figure 2 (a-c), of samples of FDF (42h/3kgf) show that accelerated

Table 1. Physical properties of the diatomaceous earth used in this study. PSD = Particle size distribution [μm], SA = Surface area [m^2/g].

NFF No.	PSD			SA
	D10	D50	D90	
131-11	4.86	13.31	26.56	33.0
133-16	0.53	1.29	2.79	33.1
133-17	1.09	4.58	12.55	30.5

Table 2. Bulk Chemistry (normalised to 100%, excluding loss on ignition (LOI)) of the diatomaceous earth used in this study.

NFF No.	Na_2O	MgO	Al_2O_3	SiO_2	P_2O_5	SO_3	Cl	K_2O	CaO	TiO_2	MnO	Fe_2O_3	LOI
131-11	0.36	0.20	2.32	94.9	0.03	0.15	-	0.11	0.85	0.11	0.02	1.0	4.1
133-16	0.3	0.21	2.45	95.6	0.03	0.12	-	0.1	0.45	0.11	0.01	0.89	3.7
133-17	0.3	0.20	2.44	95.0	0.03	0.12	-	0.13	0.74	0.11	0.02	0.87	3.9

Table 3. Manufacturing rubric for sintered biosilica.

Number of samples	Sintering Force (N)	Sintering temperature ($^{\circ}\text{C}$)	Sintering time (hours)
14	10	1100	5
14	10	1100	10
14	10	1100	24
14	30	1100	5
14	30	1100	10
14	30	1100	24
14	30	1100	42
14	30	1100	72

Figure 1. Free diatom frustules (a) cut and moulded into differently shaped FDF (b) by sintering, followed by subtractive manufacturing.

solid state diffusion between the frustule walls (from sintering) results in a connected network of frustules. The nanopores of the individual diatom frustules are very likely to be a key parameter for cellular attachment and growth [9]. In the SEM micrographs of Figure 2(d), it is clear that these frustules remain ornamented with interconnected nanopores, a feature needed for e.g. bone cell infiltration and rapid bone tissue growth.

The way in which a biomaterial is used often depends on its mechanical character alongside its physico-chemical character. Bone grafts for example, may require porosity, chemical biocompatibility, and a degree of stiffness, strength and toughness that would enable it to be used as a grafting material. Sintering time appears to have no effect on the strength, toughness and strain to failure properties of FDF. In the case of the Youngs modulus however, we no-

Figure 2. (a-d) representative SEM micrographs from a sample of FDF after sintering at 42 hours/3kgf and (d) the frustule nano-architectures remain preserved after sintering which we propose is important for cell attachment. Scale bars = 1 μm .

tice that the Young's modulus is very similar, irrespective of time, for samples sintered at 3kgf under 25 hours ranging averaged at 25.1MPa ($\pm 5.4\text{MPa}$), and for samples at 3kgf sintered over 25 hours averaged at 37.3MPa ($\pm 11.3\text{MPa}$). The Young's modulus of 1kgf FDF could not be calculated as considerable micro-cracking observed in the stress-strain curve from the onset of loading made it impossible to identify any region of linear elasticity in the stress-strain curve. We find nevertheless, the independent variable that most prominently affects the mechanical performance of FDF is the sintering pressure. The compressive strength of FDF at 1kgf and 3kgf have strength values of 0.77MPa ($\pm 0.23\text{MPa}$) and 1.42MPa ($\pm 0.45\text{MPa}$) respectively, and toughness values of 76.2kJm^{-3} ($\pm 17.2\text{kJm}^{-3}$) and 134.6kJm^{-3} ($\pm 24.7\text{kJm}^{-3}$) respectively.

4.2. FDF material as growth platform for culture cells

To test whether FDF is biocompatible with human cells, a series of in vitro biocompatibility tests were performed. The FDF was initially washed with distilled water and autoclaved. Small fragments of FDF were then placed in 6-well culture plates and covered with cell culture medium (DMEM/F12 with 10% FCS). FDF is not translucent and it would be unlikely that we could see cells attach to it without using autofluorescence staining. We therefore used HEK293 cells stably transfected with an AmCyan-P2A-mCherry construct that we have previously reported [25]. These cells (HEK-AC) glow at two distinct

wavelengths and cellular localisations (nuclei is AmCyan-positive while cytoplasm is mCherry-positive). Prior to plating the HEK-AC cells, we checked the FDF set upon a NUNC cell culture plate under the EVOS FL fluorescence microscope and confirmed that it did not fluoresce, Figure 3.

Figure 3. The Figure shows the FDF (bottom left) does not autofluoresce and thus appears black under the EVOS FL fluorescence microscope. The image is a composite (or overlay) of light emission and fluorescence imaging using green and red filters. The NUNC cell culture plate is showed in grey colour (upper right). Scale bar = 400 μm .

We plated the HEK-AC cells on the NUNC plates holding the FDF, as well as on control NUNC plates with only FDF and no HEK-AC cells. 24 hours after plating we could hardly see any cells on the plate surface of wells containing FDF under white light, Figure 4 (a), (c) and (e). However, once we switched on the fluorescence, the cells became visible and were densely packed on the primarily FDF, Figure 4 (b), (d) and (f), and sparsely on the NUNC cell culture plate. This suggested that HEK-AC cells prefer to adhere to the FDF rather than on the surface of the NUNC plates. The cells continued growing to higher densities on the FDF for the next 21 days, and only after this point did cells start growing in greater density on the NUNC plate surface.

In order to make sure the cells were alive, and that other cells types would grow to FDF, we plated MDA-MB-231 breast cancer cells in the same way as described for the HEK-AC cells. We used a viability fluorescent dye (Calcein-AM), which is non-fluorescent until metabolised by esterases in living cells, into a green-fluorescent dye. MDA-MB-231 cells also selectively attached to the FDF rather than to the plate surface, Figure 5, and after 5 days of culture we added Calcein-AM. As shown in, Figure 5(b-c), living MDA-MB-231 cells (Calcein-positive) were attached on the surface of the FDF. To ensure that the cells were not placed under the FDF, we collected fragments of the material using sterile tweezers, washed them in PBS, and place them into new plates. Visualisation of the cells under the fluorescence microscope at the later stages of development shows that the MDA-MB-231 cell structures and morphologies were preserved, Figure 5(d). Early stage and late stage HEK-AC cell growth on FDF was compared (>300 cells for each stage) using Fiji-Image-J v.1.52p. We laid particular focus on the perimeters of the cells normalised against the dimensions of the surrounding box as this was indicative of cell spread, and on the aspect ratios of HEK-AC cells as an indication of cell-health. We found that the normalised perimeter of early stage cells ($153 \mu\text{m}/\mu\text{m}$) were approximately one quarter that of later stage cells ($655 \mu\text{m}/\mu\text{m}$) indicating that later stage cells had grown and spread effectively across the FDF as a function of time. The aspect ratios of early stage cells were notably smaller on average ($2.84 \mu\text{m}/\mu\text{m}$) than later stage cells ($5.30 \mu\text{m}/\mu\text{m}$), indicating that cell-structures had developed healthily from the early to the late stages. In order to visualise the attachment of HEK-293T cells to the FDF, we used probes labelling the cell cytoskeleton and mitochondria (labelling the actin plus nuclei, or mitochondria plus nuclei, respectively). Figures 5(e-f) are representative images of HEK-293T cells growing on the FDF whilst retaining their correct morphologies, an indication of good cell-health.

4.3. FDF is a reusable cell culture platform

We tested if the FDF could be reused as this would make FDF more useful through increased longevity. We thence grew HEK-AC cells on it for 21 days (as above)

and visualised under the microscope that there were cells on the FDF fragments. These same fragments were then either 1) washed with 96% ethanol for 10 minutes, following a series of washings with PBS, or 2) washed with dH_2O and autoclaved. In both cases, no fluorescent cells could be detected after sterilisation. The same fragments were thereafter placed in 6-well plates and MDA-MB-231 cells were seeded onto them. The newly attached cells were monitored for up to 15 days using Calcein-AM staining, showing that the material is reusable and its reuse had no observed effect on cell survival.

4.4. FDF: an exemplar growth platform for bone formation

Since one of the potential uses of this material is bone-grafting, an *in vitro* bone formation test was performed where pre-osteoblasts (MC3T3 cells) were stimulated for osteogenic differentiation and mineralisation. For this, flat fragments of FDF were selected, washed and autoclaved. FDF was then plated in 6-well plates with complete alpha minimum essential medium (a-MEM) cell nutrient media until near confluency. The medium was then replaced with osteogenic medium and changed every 2 days for the subsequent 21 days.

Pre-osteoblast MC3T3 cell viability and adherence to FDF was analysed by taking some FDF fragments out of the media using sterile tweezers, and placing them in a separate well with 2 ml of a-MEM medium conditioned with Calcein-AM for 5 minutes. Cells were then observed under the fluorescent microscope (EVOS FL). Mineralisation was analysed using the von-Kossa staining test. Culture medium was removed from the wells, after which it was rinsed with ice-cold PBS. The cells on the FDF were then fixed in cold 10% neutral formalin buffer (NFB) for 15 minutes. After fixation, the fixative was removed, and the FDF fragments washed with PBS and completely covered with sterile dH_2O and stored at 4°C until further use.

The von-Kossa assay detects the formation or mineralisation of bone or mineral nodules and normally results in brown to black staining. Many brown/black stains were observed to form on the FDF samples cultured with osteoblasts, Figures 6 (a-b), whereas no phosphate deposits (brown/black) spots were observed on the negative control samples, Figures 6 (c-d). The mineralisation detected by this method is a standard procedure for determining the differentiation of osteoblasts and their activity as bone forming cells. All samples were tested using 3 biological replicates.

5. Discussion

Diatomaceous earth is the raw material used to make FDF. Diatom frustules are abundantly available in large-scale deposits around the world (diatomaceous earth), which have been mined in large volumes since the 1800s. They are a natural, sustainable and renewable material resource, offering an economic eco-friendly alternative to

Figure 4. Preferential growth of kidney HEK cells on chunks of fused bio-silicate diatom material over the cell growth plate at 15d of cell culture. Larger chunk of fused bio-silicate material appears black in (a), (c), (e) in transmission light microscope (red arrows), have high densities of kidney HEK cells growing on them as verified in (b), (d) and (f) (blue arrows) (glowing AmCyan green in the nuclei in (b) and (d)) and (glowing red as mCherry fluorescent protein in the cytoplasm in (f)), as compared to fewer cells on the commercial NUNC-cell growth plate (Sigma-Aldrich) (orange circles) or no cells at all in areas (yellow arrows). All images were taken with an EVOS FL fluorescence microscope (Thermofisher Scientific). Scale bar = $200\mu\text{m}$ in all images.

synthetic cell growth platforms and indeed bone substitutes. Two frequently used synthetic bone graft substitutes include; demineralised bone matrix (brand name DBX) and beta-tricalcium phosphate (TCP). These materials cost on average 750USD and 1100USD per case, respectively (bone substitute cost only). Other synthetic

bone graft substitutes include a $25\times 100\times 4\text{mm}$ Vitoss scaffold foam material from Orthovita costing ca. 1,380USD, and 10 mls Norian bone void filler from Synthes costing ca. 2,315USD (Bostrom and Seigerman, 2005). The diatomaceous earth used herein to manufacture FDF is available in bulk and is often priced under 70USD/tonne depending

Figure 5. Selective growth of breast cancer cells on chunks of FDF (in black) over the cell growth plate (in grey). The fused material looks dark (in all images) while cells glow green having being stained (green) with cell viability dye Calcein-AM in (b-d). In (b) we see the early stages of attachment, in (c) a point where the cells are affixed to the FDF surface, and in (d) a later stage where the cells have proliferated on the FDF. The structure/morphology of the MDA-MB-231 cells are clearly preserved after attachment to the FDF as seen most clearly in image (d). Scale bar in images (a-d) = $400\mu\text{m}$. Confocal images of HEK-293T cells growing on the material: (e) cells stained with Alexa fluor 488 phalloidin (actin F) and (f) expressing CIB1 fused with mitochondrial targeting signal and GFP (mitochondria). NucBlue staining was used for nuclear labelling. Scale bar in images (e-f) = $20\mu\text{m}$

on the grade and quality.

FDF can be moulded and/or subtractive manufactured

into different shapes (in both 2D and 3D) since the sintering process used herein forms a lightly bound porous

Figure 6. Representative images (A and B) of samples of stained mineral deposits (dark brown to black spots, von-Kossa staining procedure) on FDF after 21 days of cell culturing (post differentiation stage) with MC3T3 osteoblast cells using a Carl Zeiss Lumar V12 microscope (AxioCam ICc 3 camera). The same material, not in culture, does not stain positive with the von-Kossa staining procedure in Images C and D (control). Images scale bar = 1mm.

structure that can be easily machined (c.f. Figure 1). FDF is strong enough to be handled, washed, and autoclaved without any effects on its shape and structure. Since FDF is highly porous, it can be cleaned easily and reused to culture cells, suggesting its utility as a long-term bio-supporting platform. FDF is a non-toxic biocompatible material and supports the growth and colony growth of a variety of cell types including; osteoblasts, HEK cells and breast cancer cells. The results from HEK293 cells and breast cancer cells show that cells were able to grow on FDF with their natural morphologies, which is not always the case with other biomaterials [26]. FDF is non-toxic to cells and exhibits stronger cell adherence than surface culture plates (such as the NUNC plates used herein). This is probably due to its rough surface structure at the microscopic level. Moreover, cells are able to grow to high densities, a feature that is probably due to the high porosity of the material so cells can obtain nutrients and exchange gases via the three-dimensional structure of the material. Three different cell types were seeded on the material and in all cases cells adhered to FDF fragments far more often as compared to the plate surface.

Moreover, as a proof-of-principle that FDF can be used for bone formation, osteoblasts were differentiated and mineralised on the material. FDF has a nanoporous architecture, which provides topographical irregularity, benefiting cell-substrate adhesion, presumably through me-

chanical interlocking [27, 28]. Many hydroxyapatite bone grafts are still used as dense material [29], however, they may also be infused with pores, enabling higher levels of mechanical interlocking [30], and as such, cell growth and health. FDF is naturally nanoporous and the sintering process applied herein does not appear to affect the architectural integrity of the biomaterial. It does nevertheless, allow individual frustules to diffusion bond, which allows the structures to be post-processed, used and reused, with no observable detriment to attached and colony-forming cells. The interconnected pore network of FDF is a critical feature in many biomaterials, especially grafting materials (such as bone grafts) [31]. FDF has mechanical properties not dissimilar to many porous materials in bone grafting including; hydroxyapatite fibre reinforced poly(α -hydroxy ester) [32], polyurethane foams [33, 34], polyHIPE foams [35], HA (TTCP + DCPA)/-/Na₂HPO₄ calcium phosphate cements [36], α -tricalcium phosphate (α -TCP) powder with a foamed polysorbate 80 [37] and silicate-phosphate specular glasses [38]. These are all examples of bone graft materials with mechanical properties at the lower end of the grafting materials spectrum, however, if FDF were to be used as graft, we expect that calcification through osteoblastic proliferation, growth and maturation would strengthen and stiffen the material in a similar way to that described in [33]. Porosity is critical for the performance of bone graft materials in bone reconstruction.

Yet, most bone grafting materials are still being infused with pores through complicated time and energy consuming processes. HA bone graft substitutes, the most extensively used artificial bone graft materials [39], are still being administered in a high density form where pores are created by the use of volatile particles, water soluble porogens and/or ceramic foaming techniques among others [40]. Indeed, most 3D bioactive glass osteoconductive scaffolds are still nonporous despite their performance in bone regeneration, and their high price [41, 42]. Contrarily, diatom frustules have naturally designed 3D porous architectures, an essential parameter in bone formation and integration, which remains intact after sintering into FDF. Since the sintering of FDF requires high pressure and temperature, no organic materials remain attached to FDF, which could otherwise be a source of immune reactivity or potential contamination.

FDF has been shown herein to be biocompatible, and diatom frustules are biodegradable by modification [43]. Silica materials have been shown to be slowly degraded *in vivo*, using rabbits as models, while being replaced by bone tissue. Several clinical trials have shown that silica-derived grafts have good contact with host bone [44, 45] and these materials get reabsorbed with little local effects [46], at a speed that depends on their nanoporosity [47]. Taking this together, FDFs have similar properties and they should be tested in animal models to determine their *in vivo* biocompatibility and bio-reabsorption. The combined biocompatibility and biodegradability of FDF suggest that this material could be an effective enabler for bone growth and repair, though further studies *in vivo* would be needed to obtain evidence of this, and their ability to biodegrade *in vivo* with the growth of specialised bone resorbing osteoclast cells. As with other synthetic bone graft substitutes, FDF also circumvents problems associated with donor site morbidity and the pain that would otherwise result from autograft harvesting [42].

6. Conclusions

Diatom frustules are ubiquitous and inexpensive. Here, we demonstrate that sintering diatom frustules under specific conditions creates a solid state continuum without loss of their nano-porous architectures. We find that HEK cells, MDA-MB-231 breast cancer cells, and pre-osteoblasts grow preferentially to our biomaterial than to NUNC plates, which are manufactured as cell-culture platforms. We also find that cell structures and morphologies of attached cells are preserved during growth. We attribute the success of our biomaterial to the compatibility of biosilica with living cells, and to the nano-architectures on the surface of our biomaterial, which enable mechanical interlocking of adhesion.

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8. Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

References

- [1] LeBaron RG, Athanasiou KA (2000). Extracellular Matrix Cell Adhesion Peptides: Functional Applications in Orthopedic Materials. *Tissue Engineering*, 6:85103
- [2] Ma Z, He W, Yong T, Ramakrishna S (2005). Grafting of Gelatin on Electrospun Poly(caprolactone) Nanofibers to Improve Endothelial Cell Spreading and Proliferation and to Control Cell Orientation. *Tissue Engineering*, 11:11491158
- [3] Shin H, Jo S, Mikos AG (2003). Biomimetic materials for tissue engineering. *Biomaterials*, 24:4353-4364
- [4] Organization and Adhesive Properties of the Hyaluronan Pericellular Coat of Chondrocytes and Epithelial Cells. *Biophysical Journal*, 85:19962005
- [5] Khorasani MT, Mirzadeh H, Irani S (2008). Plasma surface modification of poly (l-lactic acid) and poly (lactic-co-glycolic acid) films for improvement of nerve cells adhesion. *Radiation Physics and Chemistry*, 77:280-287
- [6] Biomimicking Extracellular Matrix: Cell Adhesive RGD Peptide Modified Electrospun Poly(D,L-lactic-co-glycolic acid) Nanofiber Mesh. *Tissue Engineering Part A*, 12:221-233
- [7] Alam P (2017). Chapter 13: Design Considerations in the Development of Wound Healing Bio-Nanomaterials In: *Nanostructures for Novel Therapy: Synthesis, Characterisation and Applications* Ed. Grumezescu, A., 343-367, Elsevier
- [8] Lee KY, Mooney DJ (2012). Alginate: Properties and biomedical applications. *Progress in Polymer Science*, 37:106-126
- [9] Anselme K, Ploux L, Ponche A (2012). Cell/Material Interfaces: Influence of Surface Chemistry and Surface Topography on Cell Adhesion. *Journal of Adhesion Science and Technology*, 24:831-852
- [10] Oh S, Daraio C, Chen LH, Pisanic TR, Finones RR, Jin S (2006). Significantly accelerated osteoblast cell growth on aligned TiO₂ nanotubes. *Journal of Biomedical Materials Research Part A*, 78A:97-103
- [11] Milan PB, Kargozar S, Joghataie MT, Samadikuchaksaraei A (2019). Nanoengineered biomaterials for skin regeneration. In: *Nanoengineered Biomaterials for Regenerative Medicine*, 265283, Elsevier
- [12] Gulati K, Santos A, Findlay D, Losic D (2015). Optimizing Anodization Conditions for the Growth of Titania Nanotubes on Curved Surfaces. *Journal of Physical Chemistry C*, 119:1603316045
- [13] Schlie S, Fadeeva E, Koroleva A, Ovsianikov A, Koch J, Ngezhayay A, Chichkov BN (2011). Laser-based nanoengineering of surface topographies for biomedical applications. *Photonics and Nanostructures - Fundamentals and Applications*, 9:159-162
- [14] Dolatshahi-Pirouz A, Nikkha M, Kolind K, Dokmeci MR, Khademhosseini A (2011). Micro- and Nanoengineering Approaches to Control Stem Cell-Biomaterial Interactions. *Journal of Functional Biomaterials*, 2:88-106

- [15] Pelaez-Vargas A, Gallego-Perez D, Magallanes-Perdomo M, Fernandes MH, Hansford DJ, De Aza AH, Monteiro FJ (2011). Isotropic micropatterned silica coatings on zirconia induce guided cell growth for dental implants. *Dental Materials*, 27:581-589
- [16] Porte-Durrieu MC, Labrugere C, Villars F, Lefebvre F, Dutoya S, Guette A, Bordenave L, Baquey C (1999). Development of RGD peptides grafted onto silica surfaces: XPS characterization and human endothelial cell interactions. *Journal of Biomedical Materials Research*, 46:368-375
- [17] Mendes LS, Saska S, Martinez MAU, Marchetto R (2013). Nanostructured materials based on mesoporous silica and mesoporous silica/apatite as osteogenic growth peptide carriers. *Materials Science and Engineering C*, 33:4427-4434
- [18] Lopex-Alvarez M, Solla EL, Gonzalez P, Serra J, Leon B, Marques AP, Reis RL (2009). Silicohydroxyapatite bioactive coatings (SiHA) from diatomaceous earth and silica. Study of adhesion and proliferation of osteoblast-like cells. *Journal of Materials Science: Materials in Medicine*, 20:1131-1136
- [19] Medlin LK (2002). Why silica or better yet why not silica? Speculations as to why the diatoms utilise silica as their cell wall material. *Diatom Research*, 17:453-459.
- [20] Cicco SR, Vona D, Gristina R, Sardella E, Ragni R, Presti ML, Farinola GM (2016). Biosilica from living diatoms: investigations on biocompatibility of bare and chemically modified *Thalassiosira weissflogii* silica shells. *Bioengineering*, 3:35
- [21] Leone G, Vona D, Presti ML, Urbano L, Cicco S, Gristina R, Palumbo F, Ragni R, Farinola GM (2017). Ca²⁺ in vivo doped biosilica from living *Thalassiosira weissflogii* diatoms: investigation on Saos-2 biocompatibility. *MRS Advances* doi:10.1557/adv.2017.49
- [22] Cicco SR, Vona D, Giglio ED, Cometa S, Mattioli-Belmonte M, Palumbo F, Ragni R, Farinola GM (2015). Chemically modified diatoms biosilica for bone cell growth with combined drug-delivery and antioxidant properties. *ChemPlusChem*, 80:1104-1112
- [23] Cicco SR, Vona D, Leone G, Giglio ED, Bonifacio MA, Cometa S, Fiore S, Palumbo F, Ragni R, Farinola GM (2019). *In vivo* functionalisation of diatom biosilica with sodium alendronate as osteoactive material. *Materials Science and Engineering C*, 104:109897
- [24] Cicco SR, Vona D, Leone G, Presti ML, Palumbo F, Altamura E, Ragni R, Farinola GM (2017). From polydisperse diatomaceous earth to biosilica with specific morphologies by glucose gradient/dialysis: a natural material for cell growth. *MRS Communications*, 7:214-220.
- [25] Potorac I, Rivero-Muller A, Trehan A, Kielbus M, Jozwiak K, Pralong F, Hafidi A, Thiry A, Menage JJ, Huhtaniemi I, Beckers A, Daly AF (2016). A vital region for human glycoprotein hormone trafficking revealed by an LHB mutation. *Journal of Endocrinology*, 231:197-207
- [26] Jones JR (2006). Observing cell response to biomaterials. *Materials Today*, 9:34-43
- [27] Huang HH, Ho CT, Lee TH, Lee TL, Liao KK, Chen FL (2004). Effect of surface roughness of ground titanium on initial cell adhesion. *Biomolecular Engineering*, 21:9397
- [28] den Braber ET, de Ruijter JE, Smots HTJ, Ginsel LA, von Recum AF, Jansen JA (1996). Quantitative analysis of fibroblast morphology on microgrooved surfaces with various groove and ridge dimensions. *Biomaterials*, 17:10931099
- [29] Takeshita F, Ayukawa Y, Iyama S, Suetsugu T, Oishi M (1997). Histological Comparison of Early Wound Healing Following Dense Hydroxyapatite Granule Grafting and Barrier Placement in Surgically Created Bone Defects Neighboring Implants. *Journal of Periodontology*, 68:924-932
- [30] Kumar S (2009). Processing of Porous Hydroxyapatite Scaffold. MTEch thesis, NIT Rourkela
- [31] Hing KA, Best SM, Tanner KE, Bonfield W, Revell PA (1999). Quantification of bone ingrowth within bone-derived porous hydroxyapatite implants of varying density. *Journal of Materials Science: Materials in Medicine*, 10:663-670
- [32] Thompson RC, Yaszemski MJ, Powers JM, Mikos AG (1998). Hydroxyapatite fiber reinforced poly(α -hydroxy ester) foams for bone regeneration. *Biomaterials*, 19:1935-1943
- [33] Gorna K, Gogolewski S (2003). Preparation, degradation, and calcification of biodegradable polyurethane foams for bone graft substitutes. *Journal of Biomedical Research Part A*, 67A:813-827
- [34] Gianitelli SM, Basoli F, Mozetic P, Piva P, Bartulli FN, Luciani F, Arcuri C, Trombetta M, Rainer A, Licoccia S (2015). Graded porous polyurethane foam: A potential scaffold for oromaxillary bone regeneration. *Materials Science and Engineering: C*, 51:329-335
- [35] Moglia RS, Holm JL, Sears NA, Wilson CJ, Harrison DM, Cosgriff-Hernandez E (2011). Injectable PolyHIPEs as High-Porosity Bone Grafts. *Biomacromolecules*, 12:3621-3628
- [36] Zhang J, Liu W, Schnitzler V, Tancret F, Bouler JM (2014). Calcium phosphate cements for bone substitution: Chemistry, handling and mechanical properties. *Acta Biomaterialia*, 10:1035-1049
- [37] Montufar EB, Traykova T, Gil C, Harr I, Almirall A, Aguirre A, Engel E, Planell JA, Ginebra MP (2010). Foamed surfactant solution as a template for self-setting injectable hydroxyapatite scaffolds for bone regeneration. *Acta Biomaterialia*, 6:876-885
- [38] Vitale-Brovarone C, Bairo F, Bretcanu O, Verne E (2009). Foam-like scaffolds for bone tissue engineering based on a novel couple of silicate-phosphate specular glasses: synthesis and properties. *Journal of Materials Science: Materials in Medicine*, 20:21972205
- [39] Kumar P, Vinitha B, Fathima G (2013). Bone grafts in dentistry. *Journal of Pharmacy and Bioallied Sciences*, 5:125127
- [40] Sopyan I, Mel M, Ramesh S, Khalid K (2007). Porous hydroxyapatite for artificial bone applications. *Science and Technology of Advanced Materials*, 8:116123
- [41] Moore W, Graves S, Bain G (2001). Synthetic Bone Graft Substitutes. *ANZ Journal of Surgery*, 71:354-361
- [42] Liu X, Rahaman MN, Fu Q (2013). Bone regeneration in strong porous bioactive glass (13-93) scaffolds with an oriented microstructure implanted in rat calvarial defects. *Acta Biomaterialia*, 9:48894898
- [43] Maher S, Alsawat M, Kumeria T, Fathalla D, Fetih G, Santos A (2015). Microcarriers: Luminescent Silicon Diatom Replacas: Self-Reporting and Degradable Drug Carriers with Biologically Derived Shape for Sustained Delivery of Therapeutics. *Advanced Functional Materials*, 25:5240
- [44] Lindfors NC, Hyvonen P, Nyyssonen M, Kirjavainen M, Kankare J, Gullichsen E, Salo J (2010). Bioactive glass S53P4 as bone graft substitute in treatment of osteomyelitis. *Bone*, 47:212-218
- [45] Lindfors NC, Koski I, Heikilla JT, Mattila K, Aho AJ (2010). A prospective randomized 14-year follow-up study of bioactive glass and autogenous bone as bone graft substitutes in benign bone tumors. *Journal of Biomedical Research Part B*, 94:157-164
- [46] Tanner MC, Heller R, Westhauser F, Miska M, Ferbert T, Fischer C, Gantz S, Schmidmaier G, Haubruck P (2018). Evaluation of the clinical effectiveness of bioactive glass (S53P4) in the treatment of non-unions of the tibia and femur: study protocol of a randomized controlled non-inferiority trial. *Trials*, 19:299
- [47] Wheeler DL, Eschbach EJ, Hoellrich RG, Montfort MJ, Chamberland DL (2000). Assessment of resorbable bioactive material for grafting of critical-size cancellous defects. *Journal of Orthopedic Research*, 18:140-148