Use of a fluorescent probe to monitor the enhanced affinity of rh-BMP-2 to silicated-calcium phosphate synthetic bone graft substitutes under competitive conditions

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Abstract
A comparative investigation was undertaken on 1-–2 mm sized granules of two forms of synthetic bone graft substitute (SBG) with identical pore structure but varied bulk chemistry, stoichiometric hydroxyapatite (HA) and silicate substituted (0.8 wt% Si) hydroxyapatite (SA), to assess the influence of SBG chemistry on the relative affinity of an osteogenic growth factor (GF), recombinant human bone morphogenetic protein-2 (rhBMP-2). A previously described novel fluorescent probe, fluoresceinthioureidoaminocaproic acid (FTCA), was covalently attached to rhBMP-2 to give FTCA-rhBMP-2 and facilitate the quantitative monitoring of GF uptake and release from the two chemistries of SBG. The relative affinity of rhBMP-2 for the HA and SA granules was assessed at a physiologically relevant concentration of 300 ng mL-– 1 from three (increasingly complex) environments; phosphate buffered saline (PBS), minimum Eagles'' medium (MEM) and serum supplemented MEM (SCEM) in order to closely mimic clinical bone repair procedures. The results demonstrated that rhBMP-2 affinity to SBGs
was highly sensitive to both SBG chemistry and the composition of the local environment. Under the most physiologically relevant competitive conditions of SCEM, rhBMP-2 showed greater affinity to SA ($P < 0.05$) such that 50% of the rhBMP-2 in solution was adsorbed to the SA granules after only 15 minutesmin, as compared to 30% adsorbed to the HA granules. Subsequent investigation of the desorption of adsorbed GF from the SBGs demonstrated that a significantly higher percentage of the adsorbed rhBMP-2 was desorbed from HA as compared to SA granules. Together, these observations suggested that at physiologically relevant concentrations and conditions, rhBMP-2 has a greater affinity to silicate-substituted hydroxyapatite as compared to stoichiometric hydroxyapatite, which may in part explain the enhanced osteoconductivity and reported osteoinductivity for silicate-substituted hydroxyapatite based SBGs.

Keywords: Growth factor adsorption; Bone morphogenetic protein-2 (BMP-2); Fluorescent probe; Porous granules; Silicate-substituted-hydroxyapatite; Competitive binding

1 Introduction

The competitive nature of protein adsorption has been of interest within the biomaterials community and the orthobiologics industries, as understanding or control of protein adsorption and structural or conformational dependence of protein activity is key to the performance of many classes of biomedical device, while more recently the kinetics of protein adsorption and desorption

have proved critical to the design of suitable drug delivery vehicles. In the orthopaedic field this is particularly relevant when considering synthetic bone graft substitutes (SBG). SBGs are used to replace or repair diseased or damaged bone tissue due to trauma or degenerative disease such as osteoporosis, which has become of increasing socio-economic importance due to pressures on the healthcare industry resulting from the aging population and greater expectations regarding quality of life [1]. Shortage of donors and side effects remain a challenge leading to further research into approaches in tissue engineering that involves the seeding of porous biocompatible and/or biodegradable scaffolds. Understanding the nature of these scaffolds is of paramount importance, especially now, when they are no longer expected to act as passive structural matrices capable of supporting cell adhesion/proliferation, protein adsorption and such but also act as 3D carriers for the delivery of bioactive molecules to enhance tissue regeneration. [1–3]. In this study, we thus concentrated on looking into understanding and monitoring the effect of protein adsorption on our scaffolds. Previous competitive studies analysed via

different techniques and on various biomaterials, all showed that the competitive nature of proteins is strongly influenced by the protein concentration [4] affinity to the biomaterial surface [5] and time [6], where a temporal dependence can manifest itself as the “Vroman” effect, where small highly mobile proteins that may initially colonize a surface are gradually replaced by less mobile proteins with a greater affinity for the surface. It is well recognised that pore size and pore connectivity play an important role [7], however, in this instance, the focus is mainly on monitoring and understanding the protein adsorption behaviour observed from our scaffold in a competitive environment. The presence of secondary protein species has also been shown to affect adsorption of a primary protein through influence on conformational change of the primary species [8].
Recently, we developed a novel methodology to covalently attach a novel fluorescent probe to a target protein and validated the ability of the probe to monitor both the adsorption and desorption of the target protein species from a variety of environments [9]. This was in response to studies conducted within our laboratories on silicate substituted hydroxyapatite (0.8 wt% Si, SA) and stoichiometric hydroxyapatite (HA) which demonstrated that on introduction into a physiological environment apatite based synthetic bone graft substitute (SBG) materials undergo specific patterns of ionic and proteomic exchange with local physiological fluids that are sensitive to the precise chemistry of the SBG. Moreover we identified that variation in the sequestering of specific adhesion proteins, such as fibronectin and vitronectin subsequently appeared to direct the behaviour of osteogenic cells [10–15].
This is particularly interesting as studies conducted in vivo with hydroxyapatite based porous SBG scaffolds with matched pore structures but relatively small variations in chemistry (ranging from 0- to 1,5 wt% Si substitution) have demonstrated optimal bone regeneration at a level of 0.8 wt% silicate substitution, including accelerated vascularisation, an increase in the rate of early bone formation and elevated levels in the equilibrium volume of new bone formed [16–19] Moreover, studies using an ectopic animal model demonstrated that SA SBG porous granules behaved like an osteoinductive autograft with the observation of spontaneous mesenchymal and endochondral bone formation within the ectopically placed grafts, suggesting multiplicity in the mechanisms of bone formation supported by these grafts. More recently, De Santis et al. found that in vivo, a hydroxyapatite substituted with Iron (Fe-HA) showed greater cell growth leading to improved bone regeneration as compared to stoichiometric HA [20], further highlighting the complexity and sensitivity of protein adsorption and subsequent biological response to chemical modification of SBG scaffolds.
We hypothesised that there may be a chemotactic dependence on this behaviour with optimal bulk chemistries relating to optimal surface physico-chemistries that sequester and enrich key adhesion proteins, angiogenic and osteogenic growth factors to confer apparent osteoinductive behaviour on

the synthetic scaffolds. Thus, the development of a tool to facilitate the tracking of the interactions between a single species of protein and a 'real' non- idealised SBG surface under a range of environments was key to testing this theory through the investigation of the affinity between the 'optimal' formulation of 0.8 wt% Si SA and stoichiometric HA.

Bone morphogenic protein-2 (BMP-2) is a powerful growth factor (GF) involved in the process of osteogenesis. It plays a central role in osteogenic cell recruitment, proliferation and differentiation and has FDA approval for use in specific clinical indications, including treatment of open fractures of long bones, non-unions and spinal fusion, to aid bone regeneration and repair. It has been critically assessed as a mediator of bone induction, often used in conjunction with injectable hydrogels or hydrogel-nanoparticle combinations [21] for improved bone formation in various studies across the field [22–24]. It was discovered in 1965 by Urist to induce bone formation when purified from decalcified bone [25–26] and then further investigated as a member of the transforming growth factor-β (TGF-β) superfamily [27]. Its role as a regulator of new bone formation appears to be tied to its ability to act both morphologically and mitogenically and thus play a critical role in mesenchymal stem cell proliferation and differentiation. [28,29–31]. However, there are concerns associated with clinical complications related to the use of supra-physiological doses of BMP-2 as opposed to SBGs [32–33]. Moreover, there is evidence that in SBGs with highly osteoconductive or osteogenic characteristics the regulation of BMP-2 expression is key, where studies by Jenis et al. [17] into the temporal pattern of BMP-2 gene expression within tissue harvested from the central and peripheral aspects of fusion masses after postereolateral lumbar fusion on adult New Zealand white rabbits, demonstrated no significant variation in the expression pattern of BMP-2 throughout fusion masses treated with either SA or the gold standard, autograft but elevated expression of collagen I, and a greater peak expression of collagen II in the central zones of SA treated fusions. Suggesting that there is some modification of the pattern of gene expression within the bone formation cascade when exposed to SA, where SA stimulates equivalent or earlier expression of bone formation markers as compared to treatment with autograft [17]. Therefore this paper, details the investigation of the adsorption and desorption behaviour of rhBMP-2 at a physiologically active concentration of 300 ng mL-– 1 [34–35] under a series of increasingly complex environmental conditions at 37 °C to investigate the sensitivity of these processes to SBG chemistry and the local environment to test the hypothesis that the bioactivity, and apparent osteoinductive behaviour reported for SA SBG [36–37] may have a chemotactic dependence.

2 Materials and methods

All chemicals and reagents for the synthesis of the ceramic samples were obtained from VWR. Hydroxyapatite powders were produced via an aqueous precipitation route as previously described by Gibson et al. [38] and porous granules were synthesised via a novel slip foaming technique. Recombinant human bone morphogenetic protein-2 (rhBMP-2) was purchased from Sera Laboratories (UK), phosphate buffer solution (PBS), minimum Eagles'' medium (MEM), foetal bovine serum (FBS) and dialysis tubes were obtained from Sigma-Aldrich.

2.1 Labelling of protein species and test media

Labelling of the rhBMP-2 with FTCA and the preparation of FTCA-rhBMP-2 standards were made as previously described by Mafina et al. [9]. Experiments were conducted in 3 test media, (i) phosphate buffer solution (PBS), (ii) minimum Eagles'' medium (MEM) and (iii) MEM supplemented with 10% foetal bovine serum (SCEM), so as to closely mimic clinical bone repair studies; where proteins are often prepare at room temperature with a particular medical device, then implanted in physiological defects at 37o °C.

2.2 Adsorption and desorption protocol

1.5 mL of FTCA-rhBMP-2 protein solution, at a concentration of 300 ng mL-– 1, was placed in triplicate in clean glass vials containing 0.5 g of porous granules per vial. To analyse the adsorption,

100 µL aliquots of the protein solution were removed at time intervals of 1, 5, 10 and 15 min. After 15 min, the porous granules were removed, and placed in a second clean glass vial containing 1.5 mL of fresh test media and gently agitated for
30 secondss to remove any loosely bound FTCA-rhBMP-2. The porous granules were then removed and placed in a third clean glass vial also containing 1.5 mL of fresh test media to analyse desorption, which was performed through removal of aliquots of 100 µL at time intervals of 5, 60, 240 and 1440 min after the addition of the granules to the test media. For validation the test media used to wash the loosely bound protein was also analysed.
Using the procedures previously established [39] appropriate volumes of both adsorption and desorption samples containing unknown concentrations of labelled BMP-2 were pipetted in triplicate and repeated, so n = 6, into a 96-well plate and the appropriate test media added to make a final volume of 200 µL per well. Low and high concentration standard dilution series were run on each plate to prepare calibration curves as shown in Figure. 1. Fluorescence intensity of the unknowns and standards were measured using a fluorescence spectrometer (Galaxy, UK) with excitation and emission wavelengths set at 494 and 520 nm, respectively.

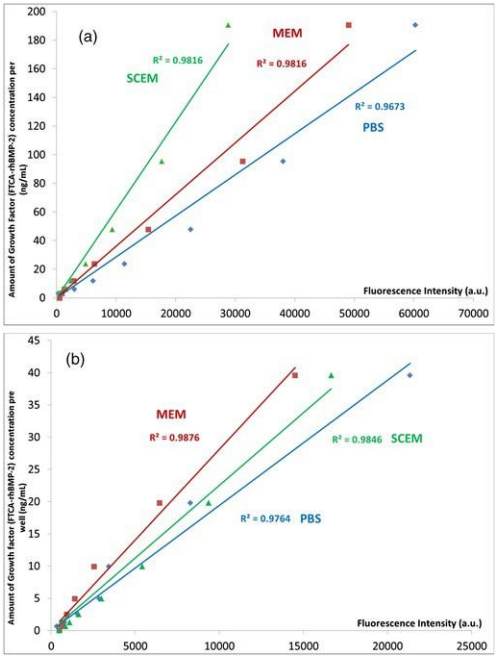

Fig. 1 Calibration curves for the (a) high and (b) low range FTCA-rhBMP-2 dilution series.

2.3 Statistical analysis
The Krusal-Wallis (KW) test was used for statistical analysis between treatment groups (media type or specimens) and to compare within the same treatment group at different time points the Wilcoxon-Mann-Whitney (WMW) was used, where for both p values of less than < 0.05 were considered statistically significant (KaleidaGraph, v8.0 Synergy software).

3 Results
FTCA-rhBMP-2 adsorption (uptake) processes

Figure. 2(a) shows a distinct variation in the adsorption behaviour of FTCA-rhBMP-2 to HA granules as the test media was varied. In PBS a plateau was not reached by the end of adsorption experiment, whereas with both MEM and SCEM FTCA-rhBMP-2 adsorption reached a maximum relatively quickly (after only 1 min) and maintained this level of adsorption throughout the length of the experiment. However, adsorption from MEM and SCEM differed in the maximal amounts of growth factor adsorbed on HA, with significantly more FTCA-rhBMP-2 (138.5 ± 7.5 ng mL-– 1, $p < 0.01$) adsorbed

onto the HA granules from MEM.

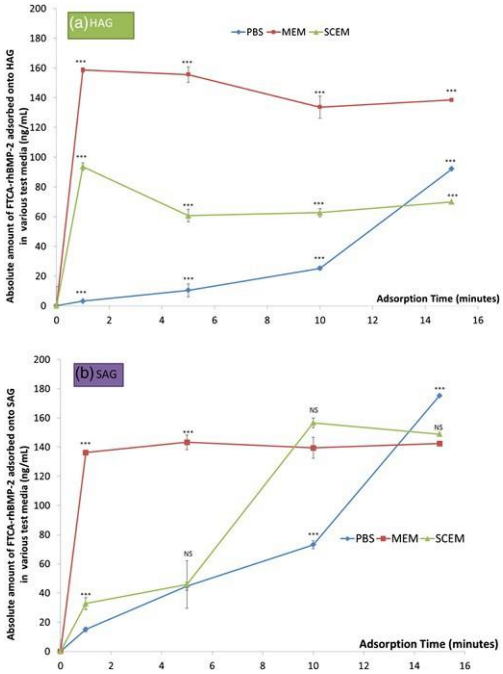

Fig. 2 Absolute amounts of the FTCA-rhBMP-2 adsorbed onto (a) HAG and (b) SAG in PBS, MEM and in SCEM at 37 o°C (n = 6 ± std.; *** corresponds to p value of < 0.005; NS denotes Not Significant).

Figure. 2(b) also shows a distinct variation in the adsorption behaviour of FTCA-rhBMP-2 to SA granules as the test media was varied. Similarly to HA, a plateau was not reached by the end of the adsorption experiment in PBS, although a significantly higher level of growth factor was adsorbed onto the SA as compared to the HA (175.2 ± 2.8 ng mL-– 1, $p < 0.01$). In MEM, the pattern of BMP-2 adsorption to SA was similar to HA, reaching a maximum after only 1 min of incubation. However, unlike the behaviour observed with HA, the maximum level of BMP-2 adsorption from SCEM to SA was not attained until after 10 min of incubation, and for SA there was no significant difference between the maximal level of FTCA-rhBMP-2 adsorbed from either MEM or SCEM (respectively 142.4 ± 7.1 or 148.9 ± 3.3 ng mL-– 1; NS).

This variation in BMP-2 adsorption behaviour from the most physiologically relevant environment (SCEM) is highlighted in Figure. 3, which shows significant variation in the adsorption behaviour of BMP-2 to HA and SA SBG. On HA, 30% of the FTCA-rhBMP-2 in solution was rapidly adsorbed to the granules within the first minute followed by a net loss in the level of adsorbed BMP-2 to 18% at 5 min and then no subsequent variation for the rest of the experiment. In contrast by 1 and 5 min only 10% and 15% respectively, of the BMP-2 had adsorbed to the SA granules from SCEM. However by 10 min this level had increased significantly to 50% of the BMP-2, with no significant variation for the rest of the experiment.

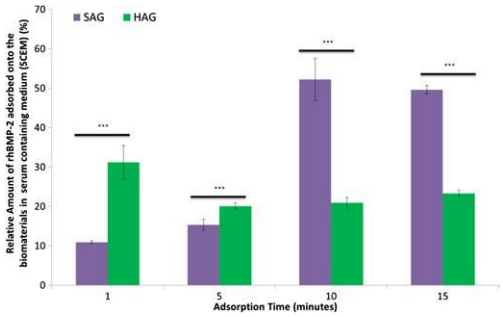

Fig. 3 Relative percentage of FTCA-rhBMP-2 adsorbed onto HAG (labelled green) and SAG (labelled purple) in SCEM at 37 o°C (n = 6 ± std.; *** corresponds to p value of < 0.005).

3.2 FTCA-rhBMP-2 desorption (release) process

Fig. 4 shows the results of the desorption experiments, conducted over a period of 24 h, demonstrating differences in behaviour with both SBG chemistry and with desorption media environments.

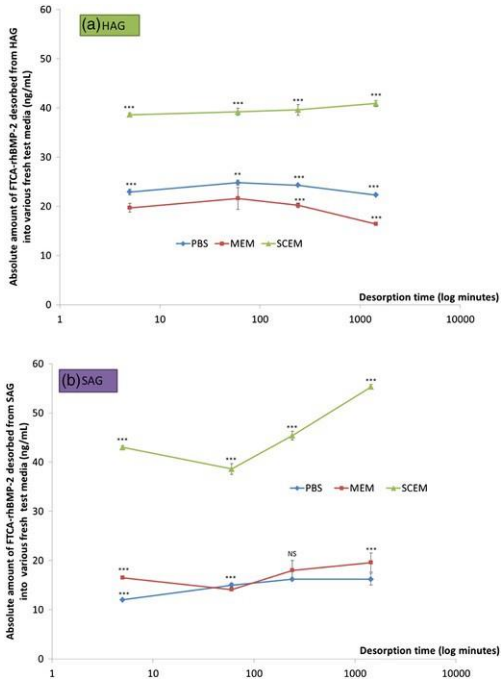

Fig.4 Desorption patterns of the FTCA-rhBMP-2 desorbed from (a) HAG and (b) SAG in PBS, MEM and in SCEM at 37 o°C (n = 6 ± std.; *** corresponds to p value of < 0.005).

On incubation in either of the serum free environments, (PBS or MEM), a significant quantity of BMP-2 was desorbed from both the HA and SA SBG within the first 5 min of incubation, after which a dynamic equilibrium appears to have become established between the rhBMP-2 desorbed into the media and the BMP-2 pre-adsorbed on the granules. The absolute quantity of BMP-2 desorbed from HA SBG was in general greater than that desorbed from SA SBG, particularly relevant when one considers that there was a greater quantity of BMP-2 initially adsorbed to the SA granules, with BMP-2 desorption from SA showing less sensitivity to the nature of the serum-free environment

and BMP-2 desorption in MEM showing less sensitivity to the SBG''s chemistry (in terms of absolute quantity of BMP-2 desorbed).

Incubation in the protein containing environment (SCEM) demonstrated that desorption of rhBMP-2 appeared to be significantly enhanced by the presence of the “competing” proteins in solution from both HA and SA SBG, and initially (after 5 min) the absolute levels of BMP-2 released from the HA and SA granules were very similar. However, the pattern and relative percentage of protein desorption was highly chemistry sensitive as shown in Figure. 5 which compares the relative percentage of growth factor desorbed into fresh GF-free SCEM.

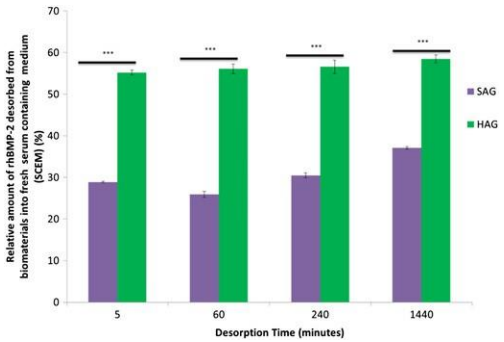

Fig. 5 Release patterns of the FTCA-rhBMP-2 desorbed from HAG (labelled green) and SAG (labelled purple) in SCEM as a percentage adsorbed at 37 o°C (n = 6 ± std.; *** corresponds to p value of < 0.005).

Within the first 5 min of incubation in SCEM; 55 % of the BMP-2 adsorbed onto HA granules was desorbed with no significant further release over 24 h. In contrast, only 30 % of the BMP-2 was released from SA granules after 5 min and over the subsequent 24 h, only a further 5 % was released.

4 Discussion

The aim of this study was to investigate the relative affinity of an osteogenic growth factor (GF), recombinant human bone morphogenetic protein-2 (rhBMP-2) for porous stoichiometric hydroxyapatite (HA) and silicate substituted hydroxyapatite (SA) synthetic bone graft (SBG) substitute granules from a series of increasingly complex environments. Our objective was to confirm or refute our hypothesis that one mechanism of action by which SA SBG may enhance new bone formation in vivo is via modification of the osteogenic response through the enrichment of key adhesion proteins and growth factors that combine to recruit both pre-committed osteogenic and stem cells and direct their differentiation down an osteogenic pathway.

More recently studies using an osteoinductive animal model to assess the combined effect of increasing strut porosity and altering graft chemistry demonstrated a synergistic relationship between silicate substitution and strut microporosity [36–37,40]. This has led to interest in the combined effect of these parameters on both protein adsorption [39,41] and ion release [42]. Investigation of protein adsorption has identified that graft chemistry affects both affinity and confirmation while ion release has recently been determined to vary significantly with strut porosity in SBG suggesting that chemotactic, biomechanical and biochemical cues combine to mediate osteoconduction and osteoinduction by multiple pathways.

No plateau of FTCA-rhBMP-2 adsorption was observed on SA or HA SBG in PBS, suggesting that the medium’'s chemistry and interactions with BMP-2 did not have a pronounced effect on the mechanisms of the growth factor’'s adsorption as compared to the SBG’'s chemistry. Differences observed between the tests were defined by the maximal amounts adsorbed, found to be higher on the silicate bone graft substitute as compared to the stoichiometric bone graft (see Figure. 2a & b, the blue lines). The patterns observed in MEM as compared to PBS for both SBG clearly suggest an influence of this medium’'s more complex chemistry on the affinity of the growth factor, as maximal

adsorption occurred within the first minute and plateaued throughout the remainder of the experiment with no significant difference between the maximal adsorbed amounts on HA or SA SBGs. In this environment, FTCA-BMP-2 adsorption appeared to be more sensitive to medium interactions, irrespective of the chemistry of the SA or HA SBG (see Figure. 2a & b, the red lines). In contrast, in the most complex environment, consisting of MEM supplemented with 10% foetal bovine serum, the observed FTCA-BMP-2 patterns of adsorption were significantly different between SA and HA SBG. On HA SBG, the pattern closely mimics that of the adsorption observed in MEM, suggesting a mechanism where the medium''s chemistry still dominated behaviour. Furthermore, the presence of serum proteins appear to have inhibited FTCA- BMP-2 adsorption to HA SBG, with only 70 ng mL-− 1 adsorbed from SCEM compared to twice as much adsorbed to HA SBG from MEM. It has been previously discussed that the amino acid components of MEM have a strong affinity

with rhBMP-2 to enhance adsorption [43], which was also observed for BSA [9]. In the absence of serum protein this was also observed in the present study for both HA and SA SBG. However, the addition of serum proteins in solution was observed to inhibit the effect of MEM enhancement on rhBMP-2 adsorption to HA SBG. This implies that the dynamic adsorption behaviour of the growth factor is more driven by a protein-protein interaction than a protein-surface interaction for HA, as compared to BSA where both of these combinations were important. Support for this hypothesis exists through the work of Uludag et al. who suggested that an increase in the protein isoelectric point (pI) in solution due to the presence of additional smaller proteins, may result in a higher retention of the growth factor in solution [44]. They further proposed that charges on the growth factor were neutralised with sodium ions to limit the BMP-2 interactions with the HA surface and allowed reversible formation of water-bridged H-bonds that changed the protein conformation. However, in this work, the charges were not neutralised, suggesting they may have been free to interact with ions and amino acids present in solution, before interacting with the biomaterial''s surfaces, to thus affect the growth factor''s conformation, as in agreement with Dong et al. [43]. This change in protein conformation or morphology was also considered in the work of Guth et al. as the proposed mechanism behind variation in cell attachment to surfaces with ostensibly the same quantity of adsorbed protein [39,41].

For SA SBG, the pattern of growth factor adsorption from SCEM closely resembled that observed in PBS, suggesting its mechanism to be synergistically influenced by the bone graft substitute''s chemistry and the medium''s chemistry (see Figure. 2 a & b, the green lines). As a result in SCEM, significantly more FTCA-BMP-2 was adsorbed on SA SBG as compared to HA SBG as shown in Figure. 3.

The desorption behaviour (see Figure. 4) closely reflected the findings of the adsorption experiments, with FTCA-BMP-2 desorption being primarily influenced by SBG chemistry in PBS, the medium''s chemistry in MEM, with a synergistic influence observed in SCEM (Figure. 5). These observations further validated the point that the affinity of rhBMP-2 to a particular SBG depends on both the local medium environment and the surface chemistry of the SBG.

In summary, this study clearly showed not only the importance of the scaffolds surface chemistry on the affinity of FTCA-rhBMP-2 to bone graft substitute scaffold materials, but also the influence of the chemistry of the local physiological environment on its adsorption behaviour. The BMP adsorption behaviours with the biomaterials in the different mediums are different. For HAG, additional protein inhibited the BMP binding; but for SAG, additional protein did not affect the final adsorption quantity. It also demonstrated that, by using a physiologically relevant concentration of rhBMP-2 labelled with a novel fluorescent probe, silicate substituted hydroxyapatite has a greater affinity for rh-BMP-2 than stoichiometric hydroxyapatite under competitive conditions. This finding supports our hypothesis that there may be a chemotactic dependence behind the enhanced osteoconductivity observed for specific bulk chemistries of bone graft substitute material, relating to these grafts possessing surface physico-chemistries that sequester and enrich key adhesion proteins, angiogenic and osteogenic growth factors to confer apparent osteoinductive behaviour on the

synthetic scaffolds.

5 Conclusions

The binding affinity of rhBMP-2 for porous silicate substituted and a stoichiometric hydroxyapatite synthetic bone graft granule was investigated from a series of increasingly complicated environments. Both the composition of the local physiological environment and the chemistry of the synthetic bone graft granules were found to impact on rhBMP-2 adsorption and desorption. Under the most physiologically relevant competitive conditions of 10% serum supplemented minimum Eagles medium, silicate substituted hydroxyapatite was found to possess a greater affinity for rhBMP-2 than stoichiometric hydroxyapatite. These observations support the hypothesis that the enhanced bioactivity of these grafts materials may in part be related to their ability to sequester and enrich key adhesion proteins, angiogenic and osteogenic growth factors to confer apparent osteoinductive behaviour on the synthetic scaffolds.

Abbreviations

SBG
synthetic bone graft

HA
hydroxyapatite

SA
silicate-substituted hydroxyapatite

GF
growth factor

FTCA
fluoresceinthioureidoaminocaproic acid

PBS
phosphate buffered saline

MEM
minimum Eagles essential medium

SCEM
serum complete Eagles medium

rhBMP-2
recombinant human bone morphogenetic protein-2

FBS
foetal bovine serum

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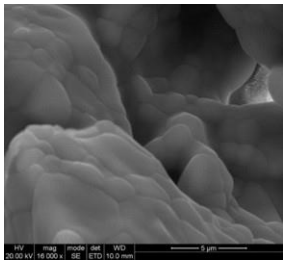
Graphical abstract
SEM image of porous granule of SA SBG gold coated.



Highlights

- describes the use of a fluorescent label covalently attached to a growth factor (RhBMP-2) to monitor adsorption and desorption behaviour of the protein on medical devices in a competitive environment.

- The binding affinity of rhBMP-2 for porous silicate substituted and a stoichiometric hydroxyapatite synthetic bone graft granule was investigated from a series of increasingly complicated environments.

- Rh-BMP-2 at a physiologically relevant concentration of 300 ngmL-1 was tested in three (increasingly complex) environments; phosphate buffered saline (PBS), minimum Eagles' medium (MEM) and serum supplemented MEM (SCEM) in order to closely mimic clinical bone repair procedures
- observations support the hypothesis that the enhanced bioactivity of these grafts materials may in part be related to their ability to sequester and enrich key adhesion proteins, angiogenic and osteogenic growth factors to confer apparent osteoinductive behaviour on the synthetic scaffolds.