Mode of heparin attachment to nanocrystalline hydroxyapatite affects its interaction with bone morphogenetic protein-2

Goonasekera, Chandhi S.; Jack, Kevin S.; Bhakta, Gajadhar; Rai, Bina; Luong-Van, Emma; Nurcombe, Victor; Cool, Simon M.; Cooper-White, Justin J.; Grøndahl, Lisbeth

2015

Goonasekera, C. S., Jack, K. S., Bhakta, G., Rai, B., Luong-Van, E., Nurcombe, V., . . . Grøndahl, L. (2015). Mode of heparin attachment to nanocrystalline hydroxyapatite affects its interaction with bone morphogenetic protein-2. *Biointerphases*, 10(4), 04A308-. doi:10.1116/1.4933109

<https://hdl.handle.net/10356/89942>

<https://doi.org/10.1116/1.4933109>

© 2015 American Vacuum Society. This paper was published in *Biointerphases* and is made available with permission of American Vacuum Society. The published version is available at: [<https://doi.org/10.1116/1.4933109>]. One print or electronic copy may be made for personal use only. Systematic or multiple reproduction, distribution to multiple locations via electronic or other means, duplication of any material in this paper for a fee or for commercial purposes, or modification of the content of the paper is prohibited and is subject to penalties under law.

Mode of heparin attachment to nanocrystalline hydroxyapatite affects its interaction with bone morphogenetic protein-2

Chandhi S. Goonasekera

School of Chemistry and Molecular Biosciences, Centre for Microscopy and Microanalysis, and Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St. Lucia, Queensland 4072, Australia

Kevin S. Jack^{a)}

Centre for Microscopy and Microanalysis, The University of Queensland, St. Lucia, Queensland 4072, Australia

Gajadhar Bhakta and Bina Rai

*Institute of Medical Biology, A*STAR, Singapore 138648, Singapore*

Emma Luong-Van

*Institute of Materials Research and Engineering, A*STAR, Singapore 117602, Singapore*

Victor Nurcombe

*Institute of Medical Biology, A*STAR, Singapore 138648, Singapore and Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232, Singapore*

Simon M. Cool

*Institute of Medical Biology, A*STAR, Singapore 138648, Singapore and Department of Orthopedic Surgery, National University of Singapore, Singapore 119074, Singapore*

Justin J. Cooper-White^{b)}

Australian Institute for Bioengineering and Nanotechnology, The School of Chemical Engineering, The University of Queensland, St. Lucia, Queensland 4072, Australia and CSIRO, Division of Materials Science and Engineering, Clayton, Victoria 3169, Australia

Lisbeth Grøndahl^{c)}

School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, Queensland 4072, Australia

(Received 17 July 2015; accepted 1 October 2015; published 16 October 2015)

Heparin has a high affinity for bone morphogenetic protein-2 (BMP-2), which is a key growth factor in bone regeneration. The aim of this study was to investigate how the rate of release of BMP-2 was affected when adsorbed to nanosized hydroxyapatite (HAP) particles functionalized with heparin by different methods. Heparin was attached to the surface of HAP, either via adsorption or covalent coupling, via a 3-aminopropyltriethoxysilane (APTES) layer. The chemical composition of the particles was evaluated using X-ray photoelectron spectroscopy and elemental microanalysis, revealing that the heparin grafting densities achieved were dependent on the curing temperature used in the fabrication of APTES-modified HAP. Comparable amounts of heparin were attached via both covalent coupling and adsorption to the APTES-modified particles, but characterization of the particle surfaces by zeta potential and Brunauer–Emmett–Teller measurements indicated that the conformation of the heparin on the surface was dependent on the method of attachment, which in turn affected the stability of heparin on the surface. The release of BMP-2 from the particles after 7 days in phosphate-buffered saline found that 31% of the loaded BMP-2 was released from the APTES-modified particles with heparin covalently attached, compared to 16% from the APTES-modified particles with the heparin adsorbed. Moreover, when heparin was adsorbed onto pure HAP, it was found that the BMP-2 released after 7 days was 5% (similar to that from unmodified HAP). This illustrates that by altering the mode of attachment of heparin to HAP the release profile and total release of BMP-2 can be manipulated. Importantly, the BMP-2 released from all the heparin particle types was found by the SMAD 1/5/8 phosphorylation assay to be biologically active. © 2015 American Vacuum Society. [<http://dx.doi.org/10.1116/1.4933109>]

I. INTRODUCTION

Bone morphogenetic protein-2 (BMP-2) is a key growth factor in bone regeneration. It is a 396 amino acid glycosylated basic polypeptide, with an isoelectric point of 8.2, consisting of two pairs of antiparallel β -strands extending from

^{a)}Electronic mail: k.jack@uq.edu.au

^{b)}Electronic mail: j.cooperwhite@uq.edu.au

^{c)}Electronic mail: l.grondahl@uq.edu.au

an α -helix that serve as surfaces for receptor interaction. It is active in its homodimeric form, which has dimensions of $70 \times 35 \times 30 \text{ \AA}$.¹ The physiological concentration of BMP-2 in blood serum of a healthy adult ranges from 25 to 100 pg ml^{-1} .² BMP-2 has been shown to improve the rate of bone healing of tissue around an implant and results in greater bone remodeling over shorter periods of time. It is believed to be involved in the initiation of the fracture healing cascade and regulation of expression of other BMPs.³ BMP-2 delivery from collagen sponges for single-level interbody spinal fusion has received regulatory approval; however, the development of products for wider clinical applications has been challenging and linked to risks, such as cystlike bone formation, osteoclast activation resulting in elevated bursts of bone resorption and potentially fatal cervical swelling.⁴ Many of these risks are due to the use of supra-physiological doses necessitated by the rapid loss of the BMP-2 from the implant surface and its labile nature ($t_{1/2} = 7\text{--}16 \text{ min in vivo}$).⁵ The currently available FDA-approved Medtronic INFUSE® Bone graft product contains 1.5 mg ml^{-1} of BMP-2.⁶ A public health notification issued by the FDA in 2008 highlighted more than 38 reports of complications resulting from the off-label use of BMP products,⁷ and a recent review on the use of BMP-2 in spinal surgeries showed that the risk of adverse side effects was 10–50 times greater than the original estimates.⁸ Therefore, it is important to develop strategies that are capable of delivering BMP-2 at dosages that minimize side effects.

Nanosized hydroxyapatite (HAP) is an osteoconductive material that is attractive for use in biocomposites for bone tissue engineering, due to its chemical and morphological similarities to bone mineral. Several studies have investigated the adsorption and release of BMP-2 from HAP as a method to create an osteoinductive biocomposite.^{9,10} However, the results achieved thus far indicate that irreversible binding of BMP-2 to HAP occurs with ensuing economic drawbacks. For example, less than 50% of the adsorbed BMP-2 was released after a period of 15 days when loaded at $70 \text{ } \mu\text{g mg}^{-1}$ onto HAP.¹⁰ Analogous behavior has been observed *in vivo*; a study conducted in a rat model demonstrated that after an initial burst-release, BMP-2 remained irreversibly bound to the inorganic bone carrier (a mixture of HAP and carbonated apatite) used in the study.¹¹ Another reason for improving HAP carriers is that the direct adsorption of BMP-2 onto the surface of HAP has been proposed to lead to conformational changes of the protein^{12,13} causing denaturation and loss of activity. Molecular dynamic simulations have suggested that the use of a monolayer of long, flexible molecules between a rigid inorganic surface and BMP-2 can prevent denaturation of the protein.¹³

A biological molecule that naturally acts as a coreceptor for BMP-2 and increases the stability of growth factor-receptor interactions is heparin/heparan sulfate.¹⁴ Heparin, a highly sulfated glycosaminoglycan (each heparin disaccharide containing 2.7 sulphate groups¹⁵), has been demonstrated to be capable of enhancing BMP-2 activity *in vitro* and *in vivo*¹⁶ and to prolong its half-life by 20-fold *in vivo*.⁵

Heparin binds to BMP-2 mainly via electrostatic interactions between its *O*-/*N*-sulfated residues and the basic amino acid residues upstream of the Transforming Growth Factor- β -type cysteine knot domain in BMP-2.¹⁷ Due to this, heparin has been used extensively for the immobilization of BMP-2 (Refs. 5 and 18–22) and has been observed to regulate the release of BMP-2.^{18,23,24} For example, heparin functionalized strontium alginate beads were observed to significantly reduce the burst-release of BMP-2 compared to nonfunctionalized beads *in vitro* and the BMP-2 released from the functionalized beads was bioactive and stimulated higher alkaline phosphatase activity in cultured C2C12 myoblast cells than freshly administered BMP-2.²⁴ Furthermore, heparin microparticles have been observed to bind BMP-2 with release of highly bioactive BMP-2 observed over 28 days.²²

The functionality of heparin has been demonstrated to be affected by how it is attached to a substrate; for example, covalently immobilized heparin has been shown to stimulate alkaline phosphatase, osteocalcin, and the differentiation of preosteoblasts to a greater degree compared to physically bound heparin.²⁵ Therefore, this study aims to investigate heparin attached to HAP in different ways. The surface modification of cerium oxide nanoparticles²⁶ and titanium²⁷ with 3-aminopropyltriethoxysilane (APTES) for the attachment of heparin has previously been reported. However, to the best of our knowledge, heparin attachment to the surface of purely ionic crystals, such as HAP, has not been conducted with the use of APTES as an intermediate molecule. In our previous study, we demonstrated that poly(acrylic acid) (PAA) can be attached both by covalent coupling and adsorption (dominated by electrostatic interactions) to the HAP surface using this method and that the conformation of PAA on the surface of HAP can be altered by the incorporation of the intermediate APTES layer.²⁸

In this study, functionalization of HAP with heparin was investigated using APTES as an intermediary linker molecule between the HAP surface and heparin. The properties of the particles with heparin attached covalently to APTES-modified HAP particles, as well as HAP and APTES-modified HAP particles with heparin adsorbed were investigated using X-ray photoelectron spectroscopy (XPS), elemental microanalysis, surface area measurements, zeta potential measurements, sizing by dynamic light scattering studies, and heparin release studies. The effect of the different modes of attachment of heparin on the adsorption, release, and bioactivity of BMP-2 is reported.

II. EXPERIMENT

A. Materials

The HAP nanoparticles and APTES-modified particles were fabricated as described previously.²⁸ The APTES-modified particles used in this study had been cured for 24 h at 150 or 200 °C and are labeled A-HAP-150 and A-HAP-200, respectively. *N*-hydroxysuccinimide (NHS, 98%), heparin sodium salt (H-3149, 181 USP u/mg from porcine intestinal mucosa), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide

hydrochloride (EDC-HCl, $\geq 98.0\%$) and 2-(*N*-morpholino) ethanesulfonic acid (MES, $\geq 99\%$) were obtained from Sigma-Aldrich. NaOH solutions were prepared using NaOH pellets (97.0%) from Pronalys. KCl ($\geq 99.5\%$) and toluene (99.9%) were obtained from Merck. Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+} , pH 7.3) (PBS) was supplied by the Agency for Science, Technology and Research (A*STAR), Singapore. Carrier-free recombinant human BMP-2 (BMP-2) was purchased from R&D systems (Minneapolis, MN), as were the BMP-2 enzyme-linked immunosorbent assay (ELISA) kits (Quantikine[®] ELISA BMP-2 Immunoassay). A heparin colorimetric assay kit [Colorimetric Assay of Heparins (UFH and LMWH) by anti- X_a method] was purchased from STACHROM[®] HEPARIN. Bovine serum albumin (BSA), basal media, Dulbecco's Modified Eagle Medium (DMEM), α -minimum essential medium eagle, and Laemmli buffer used was from Sigma-Aldrich. Glucose, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco[®]. Antibodies against phosphorylated SMAD 1/5/8 were purchased from Cell Signaling Technology. Amicon ultra-0.5 ml centrifugal filter units with a 10 K Ultracel Membrane were purchased from Millipore. The SDS-PAGE gels used were NuPAGE 4%–12% bis-tris gels from Novex Life Technologies. Deionized water (Milli-Q) water was used for preparation of all aqueous solutions.

B. Experimental setup and methodology

Modification of nanoparticles with heparin was conducted by a method based on that reported previously for modification with PAA.²⁸ Heparin (50 mg), EDC-HCl (194 mg), and NHS (289 mg) were dissolved in 5 ml of 0.05 mol l^{-1} MES buffer at pH 6.0 and stirred for 15 min at room temperature. Next, 0.1 g of A-HAP-150 or A-HAP-200 suspended in 10 ml of MES buffer was added to the above solution and stirred at room temperature for 3 h. Finally, the suspension was centrifuged at 7000 rpm for 5 min, and the supernatant was used for Ca analysis. The residue was washed three times with Milli-Q water under centrifugation at 7000 rpm for 5 min and dried at 50 °C overnight to obtain HEP-A-HAP-150 or HEP-A-HAP-200. The above steps were repeated using A-HAP-150 and A-HAP-200, without addition of EDC and NHS, to obtain HEP-A-HAP-150-ads and HEP-A-HAP-200-ads, and also carried out using HAP, without EDC and NHS, to obtain HEP-HAP.

Adsorption of BMP-2 onto the nanoparticles was done using finely ground dry particles (2 mg) that were dispersed in 0.25 ml of PBS at 4 °C by sonication for 20 s. To this suspension, 0.25 ml of BMP-2 (2000 ng ml^{-1} in PBS at 4 °C) was added, and the resulting suspension was tumbled for 2 h at 4 °C. The suspensions were centrifuged at 8000 rpm for 30 s and the precipitate was resuspended by shaking in 0.5 ml of PBS at 4 °C, followed by immediate centrifugation at 8000 rpm for 30 s. The particles were used immediately for the release study. The supernatants from both washing steps were stored at –20 °C, for up to 7 days, until a BMP-2

ELISA was done, according to the manufacturer's specifications, to quantify the total amount of BMP-2.

Release of BMP-2 and heparin was carried out as follows. A 10 mg ml^{-1} BSA containing PBS solution (herein referred to as PBS-BSA) was used as the release media for the passive release study. The BMP-2 adsorbed particles (2 mg) were suspended in 0.5 ml of PBS-BSA and tumbled at 37 °C for up to 7 days. Aliquots of 150 μl of the release media were collected at 2, 4, and 18 h, and 1, 2, 3, 5, and 7 days by centrifuging the suspensions at 8000 rpm for 30 s and collecting 150 μl of the supernatants. In order to keep the total volume constant, 150 μl of fresh PBS-BSA was reintroduced at each time point and the particles were resuspended. All aliquots were frozen at –20 °C for up to 7 days, until the amount of BMP-2 was quantified using a BMP-2 ELISA kit. To investigate the release of heparin, 2 mg each of HEP-HAP, HEP-A-HAP-150-ads, and HEP-A-HAP-150 were suspended in 0.5 ml of PBS-BSA at 4 °C, sonicated for 20 s to disperse, and tumbled for 2 h at 4 °C. The suspensions were centrifuged at 8000 rpm for 30 s and the precipitate was resuspended by shaking in 0.5 ml of PBS-BSA at 4 °C. This was centrifuged at 8000 rpm for 30 s, and the particles obtained were subjected to the same procedure described above. The amount of heparin released at each time point was determined using the heparin colorimetric assay by the anti- X_a method, according to the manufacturer's specifications.

Determination of the biological activity of BMP-2 was done using phosphorylation of SMAD 1/5/8, which is a molecular event that monitors the intracellular response to BMP-2 signaling. It was used to evaluate the bioactivity of BMP-2 released from the particles as described in a previous publication.¹⁷ C2C12 myoblast cells were seeded in 24-well tissue culture plates (40 000 cells/well) and cultured overnight in basal media, DMEM supplemented with 1000 mg l^{-1} of glucose, 10% FBS, and 1% penicillin/streptomycin. The following day, the culture media were replaced with treatment media (basal media containing 1000 mg l^{-1} of glucose, 5% FBS, and 1% penicillin/streptomycin) and cultured for 24 h. Each group of BMP-2 adsorbed nanoparticles (2 mg) was allowed to release BMP-2 in 0.25 ml of PBS supplemented with BSA (1% w/v) for 7 days. The released media from each nanoparticle group were collected after 7 days. After maintaining the cells in treatment media for 24 h, an equal volume of the released media (50 μl) collected from each group was administered onto the cells and incubated for 15 min, after which the cell layer was lysed in Laemmli buffer, resolved in 4%–12% SDS-PAGE gels (Life Technologies), and immunoblotted with antibodies against phosphorylated SMAD 1/5/8 and actin (as an internal control). Media alone and media containing 30 ng ml^{-1} of fresh BMP-2 served as the negative and positive controls, respectively.

C. Characterization

Elemental microanalysis was carried out on a Carlo Erba—NA 1500 CNS analyzer as described previously.²⁸

The elemental compositions of the sample surfaces were determined with a Kratos Axis ULTRA XPS as described previously.²⁸ Survey (wide) scans of the sample were taken with a pass energy of 160 eV with 0.2 eV steps and a dwell time of 100 ms. Charge correction was applied by setting the observed value of C 1s to be at 285 eV. The atomic concentrations were determined by deconvolution of the resulting spectra using a least-squares optimization routine in CASAXPS software. Details of the curve fitting procedure as well as curve fitted C 1s spectra are given in the supplementary material (including Fig. S1).²⁹

Transmission electron microscopy (TEM) was carried out on a JEOL 1010 TE microscope operating at an accelerating voltage of 100 kV. The specimens were prepared by taking 10 mg ml⁻¹ samples in water and evaporating them onto holey carbon coated copper grids.

The zeta-potential and hydrodynamic radii of the particles were measured using a Malvern Zetasizer NanoZS and a Malvern Zetasizer 3000HS instrument, respectively, as described previously.²⁸ Each measurement consisted of the sum of at least five coadded correlation plots, and the final value obtained was taken from the average of three replicate measurements.

The specific surface area of particles was analyzed by nitrogen Brunauer–Emmett–Teller (BET) adsorption/desorption isotherms at -196 °C on a Micromeritics Tristar II 3020 system. The samples were outgassed at 80 °C overnight before analysis was performed. The molecular radius of nitrogen was taken to be 0.162 nm². The BET constant or *C*-value is determined directly from the BET analysis of the adsorption isotherm and is related to the enthalpy of adsorption of the first layer of gas molecules³⁰ as defined in the below equation:

$$C \cong e^{(\Delta H_{\text{ads}} - \Delta H_{\text{cond}})/RT}, \quad (1)$$

where ΔH_{ads} is the enthalpy of adsorption of nitrogen gas onto the surface and ΔH_{cond} is the enthalpy of condensation of nitrogen.

Inductively coupled plasma optical emission spectroscopy (ICP-OES; 1000 W Varian Vista Pro ICPOES instrument) was conducted to determine the concentration of calcium in the supernatant of solution from the preparation of HEP-HAP, HEP-A-HAP-150-ads, and HEP-A-HAP-150. The calcium content in HAP and A-HAP-150 suspensions in MES buffer was also determined by stirring 0.1 g sample in 15 ml of 0.05 mol l⁻¹ MES buffer at pH 6.0 at room temperature for 3 h, before being centrifuged at 7000 rpm for 5 min to obtain the supernatant for Ca analysis.

D. Statistical analysis

Characterization of the samples was conducted using at least three replicate samples in all techniques. The results are expressed as mean $\pm$ standard deviation, except where the standard deviation is less than the instrumental error, in which case the result is expressed as mean $\pm$ instrumental error. Statistical analysis was done using a two-tailed

student's *t* test. A probability of $p < 0.05$ was considered to be statistically significant.

III. RESULTS AND DISCUSSION

A. Chemical characterization of nanoparticle powders

The characterization of HAP, A-HAP-150, and A-HAP-200 were detailed in our previous publication²⁸ and only a brief summary will be given here. The elemental composition of these particle surfaces was determined with the use of XPS. Analysis of HAP revealed the presence of Ca, P, and O in agreement with the chemical formula [Ca₅(PO₄)₃OH] and a C 1s peak from “adventitious carbon”³¹ [see Fig. 1(A-a)]. Modification of these samples with APTES led to the observation of Si and N in the XPS spectra [see Fig. 1(A-b)] at concentrations of 2.4 at. % Si and 0.8 at. % N for A-HAP-150 and 1.9 at. % Si and 0.8 at. % N for A-HAP-200.²⁸ In our previous work,²⁸ it was determined that significant oxidation of the APTES amine groups occurred with postreaction curing at ≥ 150 °C resulting in 1.7 and 0.4 at. % amine and protonated amines on the surface of A-HAP-150 and A-HAP-200, respectively, the remainder being amides. A representative diagram of the functional groups present on the surfaces of HAP, A-HAP-150, and A-HAP-200 are shown in Fig. 2.

Several types of heparin-modified particles were investigated in the present study. Heparin was adsorbed to the surface of HAP and the APTES-modified HAP particles. In addition, heparin was covalently coupled to the APTES-modified

FIG. 1. (A) XPS survey scans and (B) TEM images obtained for (a) HAP, (b) A-HAP-150, and (c) HEP-A-HAP-150 (the scale bar on the images is 500 nm).

FIG. 2. Schematic representation of the functional groups present on the surface of HAP, and, surface groups on A-HAP-150 and A-HAP-200 (Ref. 28) (figure not drawn to scale).

HAP particles. XPS analysis of the heparin-functionalized nanoparticles revealed that the Ca/P ratios were not significantly different to each other and were within error of the theoretical value of 1.67 for hydroxyapatite (data not shown). In agreement with the chemical structure of heparin containing sulfate groups, the attachment of heparin to the particles resulted in the appearance of a S 2p peak of all heparin-functionalized particles; the HEP-A-HAP-150 sample is displayed in Fig. 1(A-c) as an example. TEM analysis of the particles showed that the modification of HAP or APTES-modified HAP with heparin did not lead to any significant change in size or shape of the particles (average length and width were 180 ± 80 and 30 ± 10 nm, respectively, see Fig. 1(B), supplementary Fig. S2 and Table S1²⁹).

Elemental microanalysis was used to determine the amount of heparin in each sample based on the mass percentage of sulphur (data given in Table I). The amount of heparin in HEP-A-HAP-150-ads and HEP-A-HAP-150 was significantly higher than in HEP-HAP ($p < 0.01$ in both cases), indicating a higher graft density of the heparin when reacted with A-HAP-150. The amount of heparin of HEP-HAP was, in turn, significantly higher than that of HEP-A-HAP-200-ads or HEP-A-HAP-200 ($p < 0.01$ in both cases). There was, however, no significant difference between the amounts in HEP-A-HAP-200-ads and HEP-A-HAP-200 ($p = 0.88$) or between HEP-A-HAP-150-ads and HEP-A-HAP-150 ($p = 0.64$). Although the amount of sulfur observed in the XPS survey scans of the different types of heparin-modified particles was not significantly different (see Table I),

deconvolution of the high resolution C 1s scans facilitated the quantitative evaluation of the relative amount of heparin on the samples. The ratio of the carbon bound to two oxygen atoms, to the total oxygen in the sample ($\text{O}-\text{C}-\text{O}:\text{total O}$) was evaluated as an indication of the relative amount of heparin present on the surface and the results are shown in Table I (curve fitted spectra displayed in supplementary Fig. S1²⁹). It was observed that this ratio was highest for the samples HEP-A-HAP-150-ads and HEP-A-HAP-150, lowest for the samples HEP-A-HAP-200-ads and HEP-A-HAP-200, while the ratio of sample HEP-HAP was intermediate, which is in agreement with the microanalysis data. Two independent measurements thus established the same significant trends with respect to the amount of heparin on the nanoparticles.

The observation of higher graft yields on the A-HAP-150 sample compared to the A-HAP-200 sample, correlates with the concentration of amine groups in the precursor particles (see above)²⁸ and is consistent with both modes of attachment of heparin to the APTES-modified particles requiring interaction with amine groups; either via electrostatic interaction with the sulfate groups of heparin in the heparin adsorbed samples or through amide bond formation with the carboxylate groups of heparin in the covalently modified samples. An important implication of these results is that the use of an intermediate APTES layer cured at 150°C results in an increased graft yield of heparin, compared to directly adsorbing heparin to a HAP surface and this will be discussed further.

TABLE I. Chemical characterization of nanoparticle samples.

Sample	Mass % N	Mass % S	Mass % of heparin ^a	S 2p (%)	C 1s (%)	(O-C-O): O _{tot} ^{b,c}
Heparin	1.44 ± 0.01	9.45 ± 0.04	100	—	—	—
HAP ^d	0.18 ± 0.02	0	—	0	16 ± 2	0
A-HAP-150 ^d	0.51 ± 0.09	0	—	0	17 ± 2^b	0
A-HAP-200 ^d	0.30 ± 0.06	0	—	0	16 ± 2^b	0
HEP-HAP	0.22 ± 0.05	0.23 ± 0.02	2.4 ± 0.2	1.1 ± 0.1	25 ± 11	0.043 ± 0.004
HEP-A-HAP-150-ads	0.54 ± 0.08	0.33 ± 0.04	3.5 ± 0.4	1.2 ± 0.4	25 ± 3	0.051 ± 0.005
HEP-A-HAP-150	0.48 ± 0.13	0.35 ± 0.06	3.7 ± 0.6	1.1 ± 0.3	22 ± 2^b	0.047 ± 0.005
HEP-A-HAP-200-ads	0.36 ± 0.06	0.15 ± 0.02	1.6 ± 0.2	0.9 ± 0.1	22 ± 3	0.038 ± 0.004
HEP-A-HAP-200	0.35 ± 0.04^b	0.15 ± 0.02	1.6 ± 0.2	0.7 ± 0.3	24 ± 6	0.034 ± 0.003

^aCalculated from mass percentage of S.

^bThe error expressed is the estimated instrumental error.

^cCurve fitted C 1s spectra displayed in Fig. S1.

^dFrom Ref. 28.

B. Physicochemical characterization of nanoparticles

The surface area of a selection of the particles was evaluated through nitrogen adsorption studies which revealed that all the samples were mesoporous, displaying type II adsorption isotherms (data given in Table II and supplementary Fig. S3²⁹). Visual comparison of the isotherms showed that the quantity of nitrogen adsorbed for saturation of the surface ($p/p^\circ = 1$) was significantly larger in HEP-HAP and HEP-A-HAP-150-ads, compared to HAP, A-HAP-150, and HEP-A-HAP-150, and it was found that there was a larger variation between the replicate isotherms for these two samples, suggesting that these surfaces may be less homogeneous in nature than the others. The surface areas of HAP, A-HAP-150, HEP-A-HAP-150-ads, and HEP-A-HAP-150 are equivalent to each other within experimental uncertainties, and are comparable to the theoretical value of $46 \text{ m}^2 \text{ g}^{-1}$ which was calculated based on the particle dimensions (obtained from TEM) assuming the particles to be cylindrical. The surface area of the HEP-HAP sample, however, was significantly larger.

The BET constants or C -values, which gives an indication of the mean surface energy and surface polarity³² were determined from the nitrogen adsorption isotherms and are shown in Table II. Surfaces with surface $-\text{OH}$ groups have previously been shown to have higher surface energies and, subsequently, higher C -values.^{33,34} For example, bare silica surfaces were determined to have C -values between 100 and 150, which decreased to 40–60, when the surfaces were grafted with n -alkyltriethoxysilanes.³³ These observations are in accordance with the findings of the current study, where the C -value of HAP was significantly higher than that of A-HAP-150. The grafting of heparin onto A-HAP-150 increased the C -value, as expected due to the polar nature of the heparin molecule, but it was observed that the C -values of HEP-A-HAP-150-ads and HEP-A-HAP-150 were significantly different, which indicated that the chemical nature of these samples are different to each other, despite similar grafted yields of heparin.

Previous studies have found that a change in the surface area of HAP can be caused by surface erosion by acidic solutions and has been observed for HAP (Ref. 35) and for $\text{Ca}_3(\text{PO}_4)_2$.³⁶ Furthermore, heparin has been observed to cause calcium dissolution from HAP in previous studies,³⁷

which can be attributed to the high affinity of heparin for calcium ions [$\log K = 4.5$ (Ref. 38)]. It has been proposed that the carboxylate and sulfate groups on the heparin chain ($\text{p}K_a$ of 2.8–3.1 and 0.5–1.5, respectively³⁹) also interact by specific interaction with Ca^{2+} in the HAP lattice.^{38,40} The extent of dissolution of calcium ions was evaluated in the current study by measuring the calcium concentrations in the suspension of HAP and A-HAP-150 during the preparation of HEP-HAP, HEP-A-HAP-150-ads, and HEP-A-HAP-150. HAP and A-HAP-150 were suspended in MES buffer to determine the inherent amount of calcium dissolved into this medium, and the results displayed in Table II showed that the dissolution of calcium from HAP and A-HAP-150 was comparable. With the addition of heparin to the medium, the dissolution of calcium increased significantly; $p < 0.01$ for both HEP-HAP compared to HAP and HEP-A-HAP-150-ads compared to A-HAP-150. In the presence of EDC and NHS (in addition to heparin), a further increase in the amount of calcium dissolved was observed ($p < 0.01$ for HEP-A-HAP-150 compared to HEP-A-HAP-150-ads). However, there was no correlation between the amount of calcium dissolved from the particles and their surface area (Table II). Previous studies of materials with adsorbed polyelectrolytes have shown that changes in surface area can occur due to the altered nanoscale surface roughness.^{41–45} Such changes can be due to phase separation of the adsorbed polyelectrolyte⁴¹ or to the different conformations of the adsorbed polyelectrolyte (extended versus coiled, trains versus loops).^{42,43,45} In all cases, the behavior is strongly dependent on external stimuli such as temperature,⁴¹ ionic strength,^{41,42,44} and pH.⁴³ Specifically, for adsorption of heparin, the change in surface area (roughness) has been observed to be dependent on the chemistry of the underlying substrate.^{46–48} The extrapolation would be that the differences observed in surface area and inferred roughness in the current study are due to differences in polyelectrolyte conformation, particularly between HEP-HAP and the samples which contained an intermediate APTES layer.

The amount of heparin functionalized on the surface of the particles as a function of surface area gives a good indication of the completeness of coverage by the heparin layer. The amount of heparin adsorbed onto HAP (to form HEP-HAP) from a heparin solution concentration of 3.3 g l^{-1} was

TABLE II. Physicochemical characterization of nanoparticle samples.

Sample	Specific surface area ($\text{m}^2 \text{ g}^{-1}$)	C -value	Ca dissolution ^a (mg Ca/mg sample)	Zeta potentials ^b (mV)
HAP	47 ± 3	220 ± 30	4.7 ± 0.3	-7.4 ± 0.2
A-HAP-150	43 ± 2	50 ± 10	4.7 ± 0.1	3.0 ± 5.4
HEP-HAP	80 ± 6	150 ± 30	23.2 ± 3.2	-19.9 ± 0.4
HEP-A-HAP-150-ads	57 ± 11	230 ± 40	17.1 ± 0.5	-28.6 ± 0.4
HEP-A-HAP-150	42 ± 2	100 ± 10	26.3 ± 1.6	-23.1 ± 0.3
HEP-A-HAP-200-ads	—	—	—	-14.0 ± 0.8
HEP-A-HAP-200	—	—	—	-20.3 ± 0.4

^aAmount dissolved into 15 ml MES after 3 h.

^bDetermined after 6 min in 1 mM KCl solutions.

0.51 mg m^{-2} (based on elemental microanalysis and surface area data, Tables I and II, respectively). Previous results⁴⁹ demonstrated that the maximum amount of heparin adsorbed onto a HAP surface is $0.63 \pm 0.12 \text{ mg m}^{-2}$ and this occurs for heparin solution concentrations of $\geq 0.7 \text{ g l}^{-1}$. Therefore, the result observed for the HEP-HAP in the current study indicates that maximal adsorption of heparin has occurred. Assuming adsorption of heparin solely in “train” conformation on the particle surface and allowing a $3 \text{ \AA}$ separation for hydrogen-bonding⁵⁰ between molecules, the surface area occupied by each heparin decamer⁵¹ can be calculated to be approximately $723 \text{ \AA}^2$; therefore, 0.65 mg m^{-2} of heparin would be required to form a complete monolayer on the surface. Considering that the conformation of heparin is likely to contain “loops” and “tails” in addition to trains, these results imply that the surface of HEP-HAP, although containing the maximum amount of heparin adsorbed, is most likely patchy with areas of HAP being exposed. Comparatively, the amount of heparin grafted in the HEP-A-HAP-150-ads and HEP-A-HAP-150 samples were significantly higher (0.86 and 0.81 mg m^{-2} , respectively) than that of the HEP-HAP sample and thus the underlying substrate is less visible to surrounding species in these samples.

The zeta potentials of HAP, A-HAP-150, and A-HAP-200 in 1 mM KCl at 25°C at neutral pH within the first 6 min of dispersion were determined to be -7.4 ± 0.2 , 3.0 ± 5.4 , and $4.5 \pm 2.0 \text{ mV}$, respectively.²⁸ Upon functionalization with heparin, the zeta potential values were observed to become more negative with values given in Table II. This correlates with the introduction of the sulfated chains and is in agreement with previous studies.³⁷ The zeta potentials of the samples in which heparin was adsorbed followed the trend of increasing negative zeta potential with increasing amount attached (numerical values followed the order HEP-A-HAP-150-ads > HEP-HAP > HEP-A-HAP-200-ads). In addition, it was observed that the zeta potentials of HEP-A-HAP-150-ads compared to HEP-A-HAP-150, as well as HEP-A-HAP-200-ads compared to HEP-A-HAP-200 were significantly different despite the

same heparin surface coverage. This indicates that the conformation of heparin on the surface is dependent on the mode of attachment (whether by adsorption or covalent coupling) and highlights that all particle samples of the current study are distinctly different in agreement with the BET results. The hydrodynamic radii of the heparin-modified particles (from dynamic light scattering) ranged from 170 to 250 nm after 24 h in solution. This size is comparable to the particle sizes observed using TEM, as shown in Fig. 1(B) and supplementary Fig. S2.²⁹ In contrast, the HAP and A-HAP-150 samples displayed particle sizes greater than $1 \mu\text{m}$ within a minute of suspending in solution, indicating particle aggregation. Thus, the functionalization of the surface with heparin imparts improved colloidal stability to the particles.

C. Release of heparin from the particles

The release of heparin from HEP-HAP, HEP-A-HAP-150-ads, and HEP-A-HAP-150 was evaluated in PBS-BSA at 37°C over a 7-day time period (see Fig. 3). No significant heparin release occurred as a result of the pretreatment step mimicking BMP-2 adsorption (determined using elemental microanalysis, data not shown); the heparin modified particles thus had starting heparin concentrations as reported in Table I. After the 7-day period investigated, it was found that the percentage of heparin released was greatest from HEP-A-HAP-150-ads ($81 \pm 7\%$ of the amount of heparin initially present on the surface), and approximately 2.5 times greater than that released from HEP-A-HAP-150 ($31 \pm 5\%$). This is in agreement with previous studies that have shown that the immobilization of molecules by covalent bonding reduces desorption^{25,52} and illustrates that not only is the surface chemistry different (concluded from the BET and zeta-potential data) but also the chemical stability of the modified particles. By comparing the release of heparin from samples HEP-HAP and HEP-A-HAP-15-ads, where heparin had been adsorbed to the particles, it can be observed that heparin has a much higher affinity for hydroxyapatite compared to APTES-modified hydroxyapatite. The percentage of heparin

FIG. 3. Release of heparin from the nanoparticle samples expressed as (a) percentage cumulative release and (b) mass of heparin remaining on the particles (relative to 2 mg nanoparticles). Studies were done in PBS-BSA at 37°C . The samples included are (■) HEP-HAP, (○) HEP-A-HAP-150-ads, and (●) HEP-A-HAP-150. The dotted lines are a guide to the eye. Error bars represent standard deviations, $n = 3$, $*p < 0.05$.

released after seven days from HEP-A-HAP-150 was comparable to that of HEP-HAP ($29 \pm 3\%$), and the release profiles were significantly different only at the two initial time points measured. The shape of the release curves indicated that the mechanisms of heparin release from HEP-HAP and HEP-A-HAP-150 were different; however, since both the underlying surface and the mode of attachment differ in these two samples, it is difficult to evaluate the reason for this difference.

In a previous study by Rai *et al.*,³⁷ it was determined that the amount of heparin released from HEP-HAP particles was dependent on the media used, with the amounts being released into water or simulated body fluid being significantly different from that released into PBS. The amount of heparin released into PBS at 37 °C was determined to be 70% after 2 days and this is considerably higher than that observed in the current study (30% released after 2 days in PBS-BSA). The inclusion of BSA in the release solution in the current study is based on it being the most abundant protein in blood (55%–60% of serum protein⁵³), and thus, its presence served to better mimic the composition of body fluid. Furthermore, it was added to prevent adsorption of the released BMP-2 to the surface of the containers. Intermolecular interactions between BSA and heparin have been observed in solution,⁴⁹ and furthermore, BSA has a

strong binding affinity to HAP (Ref. 54) through its ability to interact with surface-bound calcium ions⁵⁵ and these attributes of BSA are likely to have influenced the release of heparin.

In addition to the cumulative percentage release displayed in Fig. 3(a), the mass of heparin remaining (relative to 2 mg nanoparticles) at various time points was evaluated to account for the different starting amounts of heparin (Table I), and this is displayed in Fig. 3(b). This representation of the data clearly illustrates that all three particle types have distinctly different amounts of heparin remaining at the particle surface at the later time-points. Thus, after 7 days, the particles which showed release of approximately 30% had 50 μg heparin (HEP-A-HAP-150) and 35 μg heparin (HEP-HAP) remaining while the particles that displayed a 75% release (HEP-A-HAP-150-ads) had 15 μg heparin remaining.

D. Release of BMP-2 from the particles

We chose to use a low loading of BMP-2 for our study (500 ng of BMP-2 per 2 mg of nanoparticle) since the overall aim is to develop strategies that are capable of delivering BMP-2 at dosages that minimize side effects as discussed in the introduction. The adsorption of BMP-2 to the nanoparticles was found to be $99.9 \pm 0.1\%$ as shown in Fig. 4(a) with no significant difference between the different

FIG. 4. BMP-2 adsorption to and release from the nanoparticle samples. (a) Mass of BMP-2 loaded onto each of the nanoparticle types; (b) percentage cumulative release of BMP-2; and (c) mass of BMP-2 remaining on the particles (relative to 2 mg nanoparticles). Studies were done in PBS-BSA at 37 °C. The samples included are (▲) HAP, (◆) A-HAP-150, (■) HEP-HAP, (○) HEP-A-HAP-150-ads, and (●) HEP-A-HAP-150. The dotted lines are a guide to the eye. Error bars represent standard deviations, $n = 3$, $*p < 0.05$. (d) Bioactivity of BMP-2 released for 7 days from the nanoparticle carrier systems, as assessed by phosphorylation of SMAD 1/5/8 in C2C12 cells. The negative and positive controls were media alone and media containing 30 ng mL^{-1} fresh BMP-2, respectively. The concentration of BMP-2 in each experiment is indicated.

nanoparticles. Theoretically, it would require 4.8×10^{15} BMP-2 molecules to completely cover the surface of the particles of the current study (e.g., surface area of $42 \text{ m}^2 \text{ g}^{-1}$) based on side-on adsorption of the BMP-2 on the particle surface and considering its molecular size⁵⁶ (e.g., each BMP-2 molecule would occupy a surface area of 20 nm^2). This is consistent with a previous study which showed a Langmuir-type adsorption isotherm for adsorption of BMP-2 to HAP with a plateau at $570 \mu\text{g m}^{-2}$.¹⁰ The mass of BMP-2 adsorbed per surface area of HAP in the current study was $3\text{--}6 \mu\text{g m}^{-2}$, which is equivalent to 1.0×10^{13} molecules adsorbed to the particles [based on BMP-2 molar mass of 30 kDa (Ref. 57)] and this amount is orders of magnitudes below the saturation level. The lack of an observable effect of the chemical modification of the nanoparticles on the amount of adsorbed BMP-2 is therefore attributed to the low concentrations used and a similar observation has previously been made.⁹

The release of BMP-2 from the nanoparticles was evaluated over a 7-day period in PBS-BSA at 37°C , and the cumulative release data are shown in Fig. 4(b). A large effect of the particle type on the release of BMP-2 was observed. The amount of BMP-2 released from HAP, A-HAP-150, and HEP-HAP was less than 5% over the 7-day period with approximately half released in the first hours. The amount of BMP-2 released from HEP-A-HAP-150-ads and HEP-A-HAP-150 were significantly higher, with 16% and 31%, respectively, being released over this period and for these particles a significant burst release of 6% and 8%, respectively, was observed in the first hours. It is thus clear that the presence of an intermediate heparin-functionalized APTES layer between the HAP surface and BMP-2 affects both the initial burst and the sustained release. The release data of both heparin and BMP-2 was fitted to a number of experimental diffusion-based models, including Higuchi and Korsmeyer-Peppas, but none were found to fit the experimental data adequately, indicating that factors other than diffusion were influencing the release of BMP-2 and heparin from the surface of HAP and similar observations have been made in previous studies.⁵⁸

In order for hydroxyapatite to effectively act as a delivery platform for BMP-2, the release from the carrier should be within a suitable time-frame, considering its requirement during the first 21 days of bone-healing.³ Furthermore, recent *in vivo* studies have demonstrated that BMP-2 has a greater regeneration potential when released with a burst followed by sustained release.^{18,59} Illustrated in Fig. 4(c) is the amount of BMP-2 remaining on the particles (relative to 2 mg nanoparticles). The slow release of BMP-2 from the control HAP in the current study is in agreement with previous studies^{10,11} and represents too slow a release for practical purposes. Furthermore, only a minor burst of less than 10 ng (per 2 mg nanoparticles) is observed for these as well as the A-HAP-150 and HEP-HAP particles. Modulation of the rate of BMP-2 release has previously been observed from a HAP/heparin/collagen composite where a reduction to 0.05% after a period of 7 days was found⁶⁰ while the

release from poly(ethylene imine) modified HAP was enhanced to 8% after 3.5 days, with a subsequent release of 0.1% per day.⁹ The findings of the current study of 16% and 31% release in 7 days coupled with a significant initial burst of 30 and 43 ng BMP-2 from the HEP-A-HAP-150-ads and HEP-A-HAP-150, respectively, are thus a large improvement from a practical perspective. Furthermore, the combined data from past studies and the current findings illustrate that not only do the chemical moieties introduced onto the HAP surface, but also the conformation of the modified layer, affect the release of BMP-2. A conformation and/or chemistry that result in relatively weak interactions allow for an appropriate rate of release of the protein.

The biological activity of BMP-2 released in 7 days was evaluated by monitoring the phosphorylation of SMAD 1/5/8, which is a precise molecular marker for the bioactivity of BMP-2. The binding of BMP-2 to the cell causes BMP receptor types I and II to associate and the complex formed thus triggers the phosphorylation of the receptor SMADs 1, 5, and 8. These complex with SMAD4 and translocate to the nucleus and alter the expression of target genes.⁶¹ In an independent experiment where BMP-2 release was performed in $500 \mu\text{l}$ of release media to simulate the release study discussed above, the BMP-2 concentrations after 7 days were found to be 4, 5, 7, 46, and 61 ng/ml for the HAP, A-HAP, HEP-HAP, HEP-A-HAP-150-ads, and HEP-A-HAP-150, respectively. These values correlate closely with those presented in Fig. 4(c). However, in order to allow for the final BMP-2 concentrations in the SMAD assay to be within the optimal concentration range a modified protocol with a reduced release media volume was applied. The BMP-2 concentrations resulting were somewhat different with the HEP-A-HAP-150-ads and HEP-A-HAP-150 samples releasing similar amounts under these conditions as shown in Fig. 4(d). From the biological activity data displayed in Fig. 4(d), it is clear that all lanes had similar intensity for the Actin band verifying that a similar amount of protein was loaded into each lane. Likewise, the total levels of SMAD 1/5/8 is similar for all lanes. Furthermore, the negative control (no BMP-2) resulted in no phosphorylation of SMAD 1/5/8 while a significant degree of phosphorylation was observed for the positive control (BMP-2 added). Importantly, the level of pSMAD 1/5/8 expressed in the nanoparticle samples increases with the amount of BMP-2 released after 7 days: The pSMAD 1/5/8 expression is thus low when BMP-2 is released from the HAP, A-HAP-150, and HEP-HAP samples, while high expression is observed when BMP-2 is released from HEP-A-HAP-150-ads and HEP-A-HAP-150 correlating with the higher amounts of BMP-2 released from these samples. This data confirms that BMP-2 released from all particle types is bioactive. In our study, we have shown that incorporating an intermediate APTES layer between the HAP surface and heparin affects both the initial burst and the sustained release of BMP-2 while allowing the growth factor to remain highly biological active thus providing a suitable delivery platform for this growth factor.

FIG. 5. Schematic representation of the proposed binding of BMP-2 to (a) HAP, (b) A-HAP-150 surface, (c) HEP-HAP surface, and (d) HEP-A-HAP-150-ads or HEP-A-HAP-150 surface and the inferred conformation of the heparin chains on the different particles.

E. Mechanistic considerations

The results of this study indicate that there is a higher affinity of the BMP-2 for the HAP, A-HAP-150, and HEP-HAP samples than for the HEP-A-HAP-150-ads and HEP-A-HAP-150 samples. We attribute this to differences in the manner that BMP-2 interacts with the different surfaces as illustrated schematically in Fig. 5 taking into account the different amounts of heparin on the different nanoparticle samples.

Considering that the theoretical saturation of the HAP surface by heparin is not achieved in the HEP-HAP sample, it is possible that the low amounts of BMP-2 applied in the current study is interacting directly with the HAP surface in this sample. Furthermore, in the A-HAP-150 sample, our previous study showed that the APTES layer formed was not stable in salt solution and that reduction of the silane functionality occurred within a few minutes,²⁸ which may have resulted in the exposure of the HAP surface. Therefore, the high affinity of BMP-2 to these surfaces can be attributed to BMP-2 interacting with HAP. Empirical and computer simulations suggest that BMP-2 binds to the surface of HAP via water bridged H-bonding and electrostatic interactions of $-\text{COOH}$, $-\text{NH}_2$, and $-\text{OH}$ groups on its polypeptide chain⁶² resulting in a dissociation constant of $2 \times 10^{-7} \text{ mol l}^{-1}$ in low salt solution (171 mM, pH 7.52),⁵⁴ thus indicating strong interactions between BMP-2 and HAP. In solution, heparin binds to BMP-2 with a measured dissociation constant of $K_d = 2.4 \times 10^{-9} - 2 \times 10^{-8} \text{ mol l}^{-1}$.^{63,64} Based on these values, it can be inferred that the strength of interaction of

BMP-2 with heparin in solution is higher than with HAP. The relatively low affinity of BMP-2 for the HEP-A-HAP-150-ads and HEP-A-HAP-150 samples observed in the current study leads to the inference that a different mode of association between BMP-2 and heparin occurs when the heparin molecule is bound to the HAP surface via an intermediate APTES layer, compared to when it is in solution and indicates that the interaction with the surface-bound heparin occurs in a suboptimal manner due to the conformation of heparin. Similar observations have been made in past studies, where the strength of interaction between adsorbed proteins and the HAP surface is weaker when the protein is adsorbed to a modified surface, resulting in the higher percentage release.⁶⁵

IV. CONCLUSION

This study has demonstrated that the method used for attachment of heparin to the surface of HAP influences the release of BMP-2. The grafting density of heparin on the APTES-modified HAP was found to be strongly dependent on the postcuring temperature used, with 150°C resulting in a surface that could retain a significantly higher amount of heparin compared to when heparin was adsorbed onto HAP, while the use of 200°C resulted in a significantly lower grafting density. Although the grafting density of heparin in samples HEP-A-HAP-150-ads and HEP-A-HAP-150 was comparable, the conformation of heparin differed (evident from BET and zeta potential measurements) as did the strength of interaction with the release of heparin being

significantly greater for HEP-A-HAP-150-ads compared to HEP-A-HAP-150. It was found that BMP-2 was strongly bound to HAP-150, A-HAP, and HEP-HAP, resulting in a rate of release considered too slow for practical use. On the other hand, the rate of release of BMP-2 from HEP-A-HAP-150-ads and HEP-A-HAP-150 was found to display an initial burst followed by sustained release, which has been proposed to be advantageous *in vivo*. Importantly, sustained biological activity demonstrated that these samples show potential for application as efficacious BMP-2 delivery vehicles in bone tissue engineering applications.

ACKNOWLEDGMENTS

The authors would like to thank Barry Wood for the XPS data and George Blazak for the elemental microanalysis data. This work was in part carried out at the Centre for Microscopy and Microanalysis, The University of Queensland node of the Australian Microscopy and Microanalysis Research Facility (AMMRF). C. Goonasekera would like to thank the Australian government and the University of Queensland for awarding an International Postgraduate Research Scholarship and a UQ Research Scholarship for funding this research. She would also like to acknowledge the School of Chemistry and Molecular Biosciences, UQ for the Arlo D. Harris Travel Scholarship to travel to Singapore and the Institute for Materials Research and Engineering, A*STAR for a living stipend.

- ¹C. Scheufler, W. Sebald, and M. Hülsmeier, *J. Mol. Biol.* **287**, 103 (1999).
- ²Y. J. Choi, S. T. Kim, K. H. Park, S. C. Oh, J. H. Seo, S. W. Shin, J. S. Kim, and Y. H. Kim, *Med. Oncol.* **29**, 582 (2012).
- ³R. Dimitriou, E. Tsiroidis, and P. V. Giannoudis, *Injury* **36**, 1392 (2005).
- ⁴J. N. Zara *et al.*, *Tissue Eng., Part A* **17**, 1389 (2011).
- ⁵B. Zhao *et al.*, *J. Biol. Chem.* **281**, 23246 (2006).
- ⁶D. Ben-David, S. Srouji, K. Shapira-Schweitzer, O. Kossover, E. Ivanir, G. Kuhn, R. Müller, D. Seliktar, and E. Livne, *Biomaterials* **34**, 2902 (2013).
- ⁷K. W. H. Lo, B. D. Ulery, K. M. Ashe, and C. T. Laurencin, *Adv. Drug Delivery Rev.* **64**, 1277 (2012).
- ⁸E. J. Carragee, E. L. Hurwitz, and B. K. Weiner, *Spine J.* **11**, 471 (2011).
- ⁹S. M. Shiels, K. D. Solomon, M. Pilia, M. R. Appleford, and J. L. Ong, *J. Biomed. Mater. Res., Part A* **100**, 3117 (2012).
- ¹⁰G. Xie, J. Sun, G. Zhong, C. Liu, and J. Wei, *J. Mater. Sci.: Mater. Med.* **21**, 1875 (2010).
- ¹¹S. R. P. Winn, H. P. Uludag, and J. O. D. S. P. Hollinger, *Clin. Orthop. Relat. Res.* **367**, S95 (1999).
- ¹²T. Utesch, G. Daminelli, and M. A. Mroginiski, *Langmuir* **27**, 13144 (2011).
- ¹³A. F. Oliveira, S. Gemming, and G. Seifert, *Materialwiss. Werkstofftech.* **41**, 1048 (2010).
- ¹⁴S. Murali *et al.*, *Biomaterials* **34**, 5594 (2013).
- ¹⁵I. Capila and R. J. Linhardt, *Angew. Chem., Int. Ed. Engl.* **41**, 390 (2002).
- ¹⁶T. Takada, T. Katagiri, M. Ifuku, N. Morimura, M. Kobayashi, K. Hasegawa, A. Ogamo, and R. Kamijo, *J. Biol. Chem.* **278**, 43229 (2003).
- ¹⁷N. S. Gandhi and R. L. Mancera, *Biochim. Biophys. Acta* **1824**, 1374 (2012).
- ¹⁸G. Bhakta, B. Rai, Z. X. H. Lim, J. H. Hui, G. S. Stein, A. J. van Wijnen, V. Nurcombe, G. D. Prestwich, and S. M. Cool, *Biomaterials* **33**, 6113 (2012).
- ¹⁹J. Zhao, C. Luo, Y. Chen, D. Wu, C. Shen, W. Han, M. Tu, and R. Zeng, *Carbohydr. Polym.* **86**, 806 (2011).
- ²⁰O. Jeon, S. J. Song, S.-W. Kang, A. J. Putnam, and B.-S. Kim, *Biomaterials* **28**, 2763 (2007).
- ²¹S. E. Bae, J. Choi, Y. K. Joung, K. Park, and D. K. Han, *J. Controlled Release* **160**, 676 (2012).
- ²²M. H. Hettiaratchi, T. Miller, J. S. Temenoff, R. E. Guldberg, and T. C. McDevitt, *Biomaterials* **35**, 7228 (2014).
- ²³X. Xu, A. K. Jha, R. L. Duncan, and X. Jia, *Acta Biomater.* **7**, 3050 (2011).
- ²⁴S. A. Abbah, J. Liu, J. C. H. Goh, and H.-K. Wong, *Tissue Eng., Part A* **19**, 350 (2013).
- ²⁵M. Gümüşderelioglu and S. Aday, *Carbohydr. Res.* **346**, 606 (2011).
- ²⁶S. R. S. Ting, J. M. Whitelock, R. Tomic, C. Gunawan, W. Y. Teoh, R. Amal, and M. S. Lord, *Biomaterials* **34**, 4377 (2013).
- ²⁷S. E. Kim, S.-H. Song, Y. P. Yun, B.-J. Choi, I. K. Kwon, M. S. Bae, H.-J. Moon, and Y.-D. Kwon, *Biomaterials* **32**, 366 (2011).
- ²⁸C. S. Goonasekera, K. S. Jack, J. J. Cooper-White, and L. Grondahl, *J. Mater. Chem. B* **1**, 5842 (2013).
- ²⁹See supplementary material at <http://dx.doi.org/10.1116/1.4933109> for detail regarding curve fitting of XPS narrow scans; TEM images of nanoparticles; measured lengths and widths of nanoparticles; nitrogen adsorption isotherms.
- ³⁰G. Fagerlund, *Mater. Constr.* **6**, 239 (1973).
- ³¹H. B. Lu, C. T. Campbell, D. J. Graham, and B. D. Ratner, *Anal. Chem.* **72**, 2886 (2000).
- ³²S. J. Gregg and K. S. W. Sing, *Adsorption, Surface Area and Porosity*, 2nd ed. (Academic, London, UK, 1982).
- ³³F. Bernardoni and A. Y. Fadeev, *J. Colloid Interface Sci.* **356**, 690 (2011).
- ³⁴S. Marcinko, R. Helmy, and A. Y. Fadeev, *Langmuir* **19**, 2752 (2003).
- ³⁵Y. Abe, Y. Okazaki, K. Hiasa, K. Yasuda, K. Nogami, W. Mizumachi, and I. Hirata, *Biomed. Res. Int.* **2013**, 626452.
- ³⁶J. Sohler, G. Daculsi, S. Sourice, K. de Groot, and P. Layrolle, *J. Biomed. Mater. Res., Part A* **92**, 1105 (2010).
- ³⁷B. Rai, L. Grondahl, and M. Trau, *Langmuir* **24**, 7744 (2008).
- ³⁸G. K. Hunter, K. S. Wong, and J. J. Kim, *Arch. Biochem. Biophys.* **260**, 161 (1988).
- ³⁹H. M. Wang, D. Loganathan, and R. J. Linhardt, *Biochem. J.* **278**, 689 (1991).
- ⁴⁰S. Chander and D. W. Fuerstenau, *Adsorption on and Surface Chemistry of Hydroxyapatite*, edited by D. N. Misra (Plenum, New York, 1984), pp. 29–49.
- ⁴¹M. Cornelsen, C. A. Helm, and S. Block, *Macromolecules* **43**, 4300 (2010).
- ⁴²R. A. McAloney, M. Sinyor, V. Dudnik, and M. C. Goh, *Langmuir* **17**, 6655 (2001).
- ⁴³S. S. Shiratori and M. F. Rubner, *Macromolecules* **33**, 4213 (2000).
- ⁴⁴R. A. Ghostine, R. M. Jisr, A. Lehaf, and J. B. Schlenoff, *Langmuir* **29**, 11742 (2013).
- ⁴⁵S. Seki, S. Tsukuda, S. Tagawa, and M. Sugimoto, *Macromolecules* **39**, 7446 (2006).
- ⁴⁶G. Li, P. Yang, W. Qin, M. F. Maitz, S. Zhou, and N. Huang, *Biomaterials* **32**, 4691 (2011).
- ⁴⁷M. M. Wan, W. J. Qian, W. G. Lin, Y. Zhou, and J. H. Zhu, *J. Mater. Chem. B* **1**, 3897 (2013).
- ⁴⁸K. R. Patel, H. Tang, W. E. Grever, K. Y. Simon Ng, J. Xiang, R. F. Keep, T. Cao, and J. P. McAllister II, *Biomaterials* **27**, 1519 (2006).
- ⁴⁹D. T. H. Wassell and G. Embery, *Biomaterials* **18**, 1001 (1997).
- ⁵⁰S. C. Wallwork, *Acta Crystallogr.* **15**, 758 (1962).
- ⁵¹B. B. Minsky, T. V. Nguyen, S. R. Peyton, I. A. Kaltashov, and P. L. Dubin, *Biomacromolecules* **14**, 4091 (2013).
- ⁵²S. Murugesan, J. Xie, and R. J. Linhardt, *Curr. Top. Med. Chem.* **8**, 80 (2008).
- ⁵³J. P. Nicholson, M. R. Wolmarans, and G. R. Park, *Br. J. Anaesth.* **85**, 599 (2000).
- ⁵⁴T. Boix, J. Gomez-Morales, J. Torrent-Burgues, A. Monfort, P. Puigdomenech, and R. Rodriguez-Clemente, *J. Inorg. Biochem.* **99**, 1043 (2005).
- ⁵⁵A. Klinger, D. Steinberg, D. Kohavi, and M. N. Sela, *J. Biomed. Mater. Res.* **36**, 387 (1997).
- ⁵⁶M. Laub, T. Seul, E. Schmachtenberg, and H. P. Jennissen, *Materialwiss. Werkstofftech.* **32**, 926 (2001).
- ⁵⁷W. J. King and P. H. Krebsbach, *Adv. Drug Delivery Rev.* **64**, 1239 (2012).

- ⁵⁸B. Palazzo, M. Iafisco, M. Laforgia, N. Margiotta, G. Natile, C. L. Bianchi, D. Walsh, S. Mann, and N. Roveri, *Adv. Funct. Mater.* **17**, 2180 (2007).
- ⁵⁹G. Bhakta, Z. X. H. Lim, B. Rai, T. Lin, J. H. Hui, G. D. Prestwich, A. J. van Wijnen, V. Nurcombe, and S. M. Cool, *Acta Biomater.* **9**, 9098 (2013).
- ⁶⁰S. Teixeira, L. Yang, P. J. Dijkstra, M. P. Ferraz, and F. J. Monteiro, *J. Mater. Sci.: Mater. Med.* **21**, 2385 (2010).
- ⁶¹G. J. Anderson and D. M. Frazer, *Nat. Genet.* **38**, 503 (2006).
- ⁶²X. Dong, Q. Wang, T. Wu, and H. Pan, *Biophys. J.* **93**, 750 (2007).
- ⁶³R. Ruppert, E. Hoffmann, and W. Sebald, *Eur. J. Biochem.* **237**, 295 (1996).
- ⁶⁴M. Kisiel, A. S. Klar, M. Ventura, J. Buijs, M.-K. Mafina, S. M. Cool, and J. Hilborn, *PLoS One* **8**, 1 (2013).
- ⁶⁵W.-H. Lee, C.-Y. Loo, A. V. Zavgorodniy, M. Ghadiri, and R. Rohanizadeh, *RSC Adv.* **3**, 4040 (2013).