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Supplementary Material

10.1302/0301-620X.103B3.BJJ-2020-0347.R1

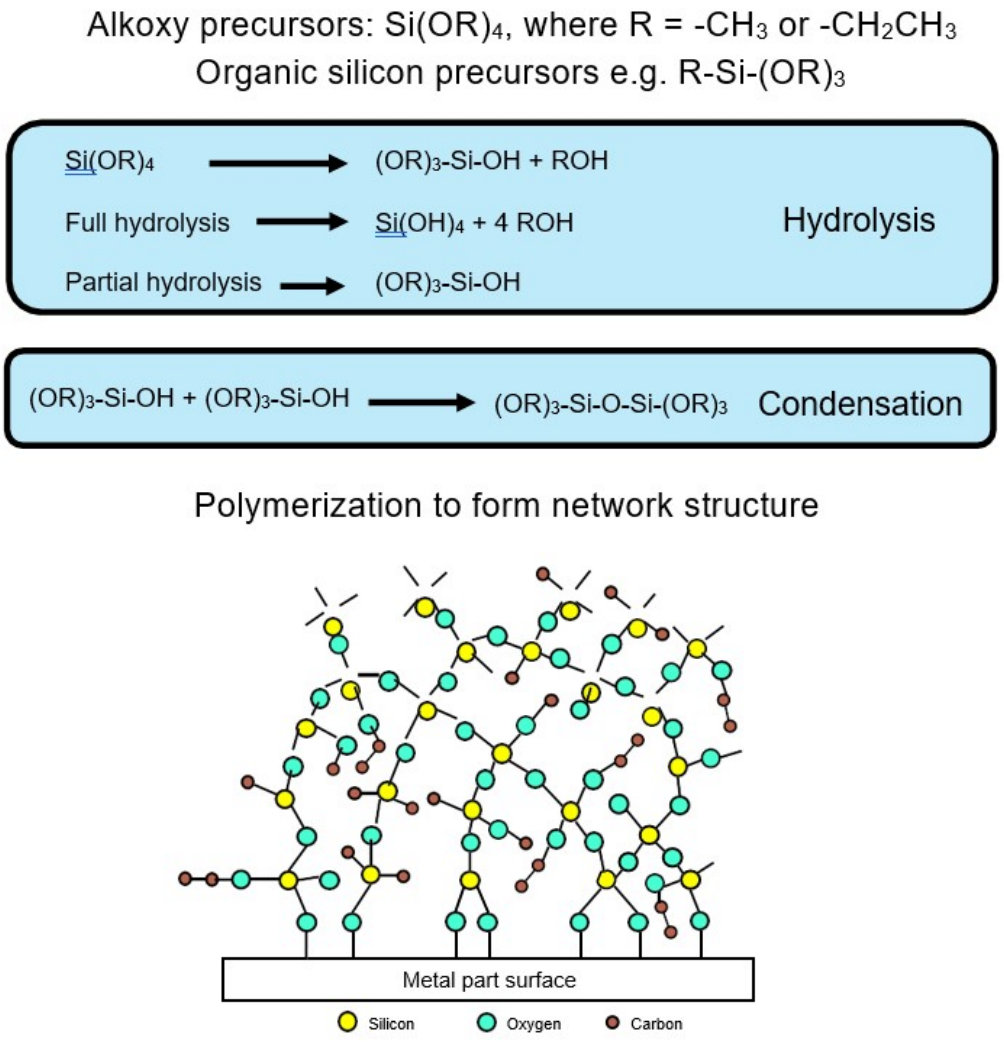

Fig. a. The sol-gel process involves colloidal particles in solution (sol), which polymerize via a series of hydroxylation and condensation reactions as shown here to form a solid porous network (gel). These can, as here, include a combination of organic and inorganic

precursor components with functional side chains designed to control the properties of the gel.

Methods

Preparation of sol-gel

The base hybrid sol was prepared using an acid catalyzed process. The precursors; tetraethylorthosilicate (TEOS), tetramethylorthosilicate (TMOS), trimethoxymethylsilane (MTMS), and poly(dimethylsiloxane) (PDMS) were stirred with isopropanol in the volumetric ratio of 0.5:1:1:0.2:4.36. The reaction was catalyzed by the addition of nitric acid (0.07M) dropwise and the sol was aged for at least 48 h. A stock solution of gentamicin was added to the sol-gel at a volumetric ratio of 0.05:0.95 to give a final concentration of 12.5 mg mL^{-1} gentamicin (equivalent on a weight/volume (w/v) basis to the antibiotic loading in bone cement). Sol-gel coatings containing gentamicin and bone morphogenetic protein-2 (BMP-2) were prepared in the same way with the addition of BMP-2 (0.5 - 2 µg mL^{-1}). The sol-gel was then vortexed for 30 s immediately prior to coating and allowed to cure for 24 h at room temperature.

In vitro elution analysis

Titanium sheet squares (20 mm × 20 mm × 0.4 mm) were coated with hydroxyapatite (50-75 µm thickness) by plasma coating (Plasma Biotal Ltd, Buxton, UK). Sol-gel coating containing the appropriate therapeutic agent(s) was applied to both the titanium-hydroxyapatite coated (Ti/HA) squares and to glass coverslips (20 mm × 20 mm) via a roller coating technique. Coated Ti/HA squares or glass coverslips were placed in 6 well plates and submerged in ammonium acetate (0.1M, pH 7.4, 5 mL) or Dulbecco's Modified Eagle Medium (DMEM), as stated for each experiment, in 6-well plates. Samples of medium containing the eluted therapeutic agents were taken over a 2-week period and stored at -20°C.

Solid phase extraction

Prior to quantification via liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS), a desalting step was required for all cell culture media samples. The samples were applied to a Strata X-CW SPE cartridge (Phenomenex) and eluted with ammonia solution (2 M in methanol). The eluate was dried under a stream of nitrogen and re-suspended in ammonium acetate (0.1M, pH 7.4). A set of gentamicin standards in DMEM were similarly prepared to provide a standard curve for quantification.

LC-MS-MS quantification of eluted antibiotic

Quantification was carried out by LC-MS-MS using a Phenomenex (Macclesfield, UK) Luna C18 column (150 mm × 1 mm) coupled to a Finnigan LCQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The isocratic mobile phase was an aqueous solution of 30% methanol, 0.2% formic acid (volume/volume (v/v)), and a flow rate of 0.05 mL min^{-1}. The mass spectrometer was operated with an electrospray ionization (ESI) source in positive ion mode with a source voltage of 4.5 kV, sheath gas flow 80 (arbitrary units), and capillary temperature 250°C. A 50% relative collision energy was used and ion 322 m/z was monitored as an ion fragment of protonated gentamicin C1 (478 m/z). Elution of gentamicin was quantified by comparison against the gentamicin standard curve described above.

Quantification of eluted BMP-2

Ti/HA squares were coated with sol-gel containing 12.5 mg mL^{-1} of gentamicin and BMP-2 at loading levels of 0.5, 1 and 2 μg mL^{-1}, and submerged in DMEM (5 mL). Samples of the eluate were taken periodically over a one-week incubation period, at room temperature. BMP-2 was quantified in the eluate samples by using the BMP-2 Quantikine ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) as per the manufacturer's protocol, in parallel with controls containing known amounts of BMP-2.

Bacterial strains and growth conditions

All strains were maintained on Mueller-Hinton agar (MH agar) or Mueller-Hinton broth (MH broth) and grown overnight at 37°C. Clinical isolates of *Staphylococcus aureus, Staphylococcus epidermidis* and *Escherichia coli* were isolated from infected prostheses at the Northern General Hospital, Sheffield, UK. *S. epidermidis* DSM 3269 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The *S. aureus* strain SH1000 was provided by Simon Foster, University of Sheffield.

Microbial kill assay

Nunc TSP-plate (Thermo Fisher Scientific) pegs were coated with the various sol-gel formulations and allowed to cure for 24 h. Staphylococcal cultures were grown overnight, diluted to a 0.5 McFarland standard and used as inoculum for each well of 96-well plates. The coated pegs were lowered into the wells of the plates so that the cultures were in contact with the coated surfaces, and the plates were incubated at 37°C, shaking at 125 rpm. After 24 h the total number of colony forming units was quantified by serial dilution and plating on MH agar plates.

Antibiofilm assay

Antibiofilm activity was determined based on the Calgary device method.[1] Staphylococcal cultures were grown overnight at 37°C, diluted to a 0.5 McFarland standard with MH broth and used as inoculum for the assay. TSP-plate (Nunc) pegs were introduced into 96 well plates containing bacterial culture and incubated at 37°C, shaking at 125 rpm, to grow biofilms. After 24 h, the biofilm coated pegs were washed in sterile phosphate buffered saline (PBS) to remove planktonic cells; then transferred into fresh MH broth in 96 well

plates in which the interior surfaces of the wells had been coated with the various sol-gel systems, and incubated for a further 24 h at 37°C, shaking at 125 rpm. The pegs were washed in sterile PBS and transferred into fresh MH broth in 96 well plates before sonicating for 10 min in a Branson 1210 Ultrasonic waterbath (Branson Ultrasonics, Danbury, Connecticut, USA) to release the biofilm bacteria into suspension. Enumeration of viable bacteria was carried out by serial dilution and plating on MH plates.

Cell culture

Rat mesenchymal stem cells (MSCs) were isolated from bone marrow from the femur of 3 rats, and cells were pooled together following extraction. MSCs were extracted using Histopaque gradient and adhesion selection prior to expansion in DMEM supplemented with fetal calf serum (10% v/v) (FCS), glutamine (2×10^{-3} mol L^{-1}), penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}). Coverslips were coated with: sol-gel alone; sol-gel containing gentamycin alone; or sol-gel containing gentamycin and BMP-2. Coated coverslips together with uncoated controls were placed in 24 well plates and 50,000 MSCs applied to the centre of each coverslip, and supplemented DMEM (2 mL) was added. The 24-well plates were maintained at 37°C in a humidified CO_2 (5%) atmosphere for 2, 7, 14 and 28 days with media changes every 3 days. (Unless otherwise stated, all media and supplements were supplied by Sigma-Aldrich (Gillingham, UK); all tissue culture plastic were supplied by Griener (Stonehouse, UK)).

Phalloidin staining

Rat MSCs adhered to sol-gel coated coverslips were washed free of media with PBS and then fixed in neutral buffered formalin (4% v/v) (NBF) in PBS. The coverslips were washed with PBS containing Tween-20 (0.05% v/v), permeabilized using Triton

X-100 (0.1%) in PBS and blocked using bovine serum albumin (1% w/v) (BSA) in PBS. After incubation for 1 h at room temperature with 1:200 tetramethylrhodamine-conjugated phalloidin (Millipore, Watford, UK) in PBS containing BSA (1% w/v). The coverslips were washed in PBS and mounted in antifade mounting solution (Millipore). Confocal fluorescence images were collected on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Cambridge, UK), excitation wavelength 570 nm.

Alizarin red staining

Rat MSCs adhered to sol-gel coated coverslips were fixed in 4% NBF and washed using PBS and stained with alizarin red solution (2 % w/v, pH 4.2) (Sigma-Aldrich, Poole, UK) for 5 minutes. Excess dye was blotted and the sample was dehydrated in acetone, cleared in xylene and mounted in a Perspex mounting medium. All slides were examined using an Olympus BX51 microscope (Olympus, Southend-on-Sea, UK) and images captured by digital camera and Capture Pro OEM v8.0 software (Media Cybernetics, Rockville, Maryland, USA).

Immunohistochemical detection of osteopontin

Rat MSCs adhered to sol-gel coated on coverslips were washed with tris-buffered saline (TBS; 20 mM tris, 150 mM sodium chloride, pH 7.5) and then non-specific binding sites were blocked for 90 minutes at room temperature with rabbit serum (25% v/v) (Abcam, Cambridge, UK) in TBS containing (1% w/v) BSA. Sections were incubated overnight at 4°C with 1:400 mouse monoclonal osteopontin antibody (Cat no. ab69498; Abcam) in TBS containing BSA (1% w/v). After washing in TBS, sections were incubated with 1:500 secondary biotinylated rabbit anti mouse antibody (Cat no. ab98784; Abcam) in TBS containing BSA (1% w/v). The bound secondary antibody was disclosed by binding

of horseradish peroxidase (HRP)-streptavidin biotin complex (30 minute incubation) (Vector Laboratories, Peterborough, UK), washing with TBS, and visualization of the bound HRP by application of 3,3'-diaminobenzidine tetrahydrochloride (0.65 mg mL^{-1}) (Sigma-Aldrich) in TBS containing hydrogen peroxide (0.08% v/v) (20 min incubation). Sections were counterstained with Mayer's haematoxylin (Leica Microsystems, Milton Keynes, UK), dehydrated in industrial methylated spirit (IMS; Fisher, Loughborough, UK) (4 × 10 minutes), cleared in SubX (Leica Microsystems) (3 × 5 minutes) and mounted in Pertex (Leica Microsystems). All slides were examined using an Olympus BX51 microscope and images captured with a digital camera and Capture Pro OEM v8.0 software (Media Cybernetics).

Implant preparation

The four implant types prepared for the in vivo model were: titanium (Ti) wire coated in hydroxyapatite (HA); Ti wire coated in HA + sol-gel (HAsg); Ti wire coated in HA + sol-gel + gentamicin (12.5mg mL^{-1}) (HAsgG); and Ti wire coated in HA + sol-gel + gentamicin (12.5mg mL^{-1}) + BMP-2 (2 μg mL^{-1}) (HAsgGB). The base material for these surfaces was Ti wire (1 mmØ) coated with HA by Plasma Biotal Ltd, to a thickness of 50-70 μm. The XYZ stage (XYZ = movable in three dimensions) of a Uniscan scanning electrochemical workstation M370 (Uniscan Instruments Ltd, Bio-Logic SAS, Seyssinet-Pariset, France) was used to further dip coat 100 mm lengths of HA coated Ti wire with sol-gel or sol-gel containing therapeutic agents, at a withdrawal speed of 10 mm min^{-1}. Prior to implantation into animals, the wires were cut to 2 mm lengths using wire cutters and then sterilized with gamma irradiation (performed by Swann Morton Ltd., Sheffield, UK).

Scanning electron microscopy

Ti/HA rods coated with sol-gel + gentamicin (12.5 mg mL^{-1}) (i.e. HAsgG) were characterized using a Phillips XL40 scanning electron microscope (SEM) (Thermo Fisher Scientific). To prevent surface charging, specimens were sputter coated with either carbon or gold and examined using secondary electron (SE) imaging and compositional analysis in backscattered electron (BSE) mode.

Surgical protocol for in vivo healing non-infection model

All work with live animals was performed at the University of Sheffield in accordance with local policies on animal use and local licences. Young adult male Wistar rats (weight 230 – 270 g) were randomly assigned to one of 4 experimental groups with 6 animals per group. Group 1: HA, Group 2: HAsg, Group 3: HAsgG and Group 4: HAsgGB. Assignment to experimental groups was performed randomly to batches (cages), with all animals within each batch treated with the same experimental group. Anaesthesia was induced and maintained with oxygen and isoflurane. Analgesia was provided by a single dose of Carprofen (Rimadyl; Pfizer, Sandwich, UK) given subcutaneously at the time of surgery. The right hind limb was extended and immobilized, and swabbed with chlorhexidine solution (0.5%). An incision was made over the medial aspect of the femur and access to the femur was by means of blunt dissection. The soft tissues were retracted and a single burr hole created in the mid-shaft of the femur using a stainless steel dental burr in a hand piece, under sterile saline for cooling; the area was further irrigated with sterile saline and a single implant placed into the defect, so that one end was within the marrow space and the other end approximately level with the cortical surface. The wound was closed with resorbable sutures (Vicryl; Ethicon, Edinburgh, UK). On completion of surgery, 100% oxygen was administered until recovery began and the animal returned to a

clean cage. For these in vivo experiments, blinding was not possible. Animals were housed in groups and maintained in normal laboratory conditions with free access to food and water for four weeks following surgery when they were sacrificed, using an approved Home Office (UK) schedule 1 method. The right femurs were dissected and placed in 4% neutral buffered formalin for fixation.

Micro-CT

Explant specimens were placed with the axis of the femur perpendicular to the plane of scanning, then scanned using a desktop microtomograph (SkyScan 1172; Bruker, Coventry, UK) through 360° at a setting 1 voxel = 6 μm. The voltage used was 70 kV, the current was 140 μA and the aluminium filter was set at 0.5 mm. The scan was collected using the camera set to medium resolution (2000 × 1048), 0.7 rotation and × 2 averaging to collect a detailed image over approximately 35 min. SkyScan allows good spatial resolution at 5.94 μm corresponding to 1×10^{-7} cubic mm voxel size. Reconstruction was carried out using NRecon (SkyScan 1172) by correcting for ring artefacts with beam hardening set at 20%.[2] A consistent anatomical position of the implant had to be taken into consideration when choosing comparable sets of data. Three samples implanted in similar positions (angle of entry and depth of insertion) were analyzed from each group. These scan data sets were used to both analyze and model each implant.

Quantitative micro-CT thresholding

Quantitative evaluations of new bone, in defined volumes of interest (VOIs), were made using CTAn software (Bruker). Each VOI was 1500 μm long by 1540 μmØ (220 μm controlled distance from the implant edge). Before segmentation, threshold levels for the implant plus coating and new bone were determined based on the visual inspection of the

histological preparation corresponding to a similar region in the micro-CT (µCT) scan. Threshold values which highlighted bone structure and the implant were measured and then applied to the whole scan VOI. This is a semi-automated technique within the CTAn. (CT-Analyser) software. It was not possible to make a clear distinction between the HA coating and the sol-gel coating, therefore the edge of the HA is designated to be equal to the edge of implant. The VOI is the tissue volume (TV) defined as the length of the implant coating × 220 µm zone around the implant edge. The percentage new bone formation (NBV) is calculated to normalize to total tissue volume within the VOI:

$$\frac{New\ bone\ formation\ (NBV)\ within\ the\ VOI}{Tissue\ Volume\ within\ the\ VOI}\ x\ 100$$

3D reconstruction

The data sets were used to produce 3D models of the implant and new bone along the coated region from a VOI of 220 μm radius around the implant. Each data set was cropped to a volume containing the implant and adjacent femur for the overall 3D model created in CTvox (SkyScan Bruker version 3). The data sets were further reduced to produce 3D models of the implant and new bone along the coated implant at a precise distance of 220 μm from the implant; the model was created in CTvol (Bruker).

Histological preparation and analysis

Following μCT imaging, the fixed specimens were dehydrated through ascending grades of ethanol, and embedded in LR white resin. The resin embedded samples were cut using an IsoMet rotary saw (Buehler IsoMet 1000; Buehler, Esslingen, Germany). The sections were attached to glass slides using Loctite glass glue (Henkel, Winsford, UK) and polished to approximately 80 μm thickness using silicon carbide grinding paper P1200 (Metprep, Coventry, UK) and aluminium oxide lapping film (Beuhler). The ground sections were stained with Stevenel's blue and Van Gieson's stains.

Digital images were captured using a light microscope (Olympus BX51 with Olympus ColourView IIIu camera and Olympus Cell D software imaging; Olympus) and quantitative evaluation of bone implant contact (BIC) percentages were calculated using Image J (Image J ver. 1.48; National Institutes of Health, Bethesda, Maryland, USA) from the entire implant collected on ×20 objective applying standard morphometrical techniques.

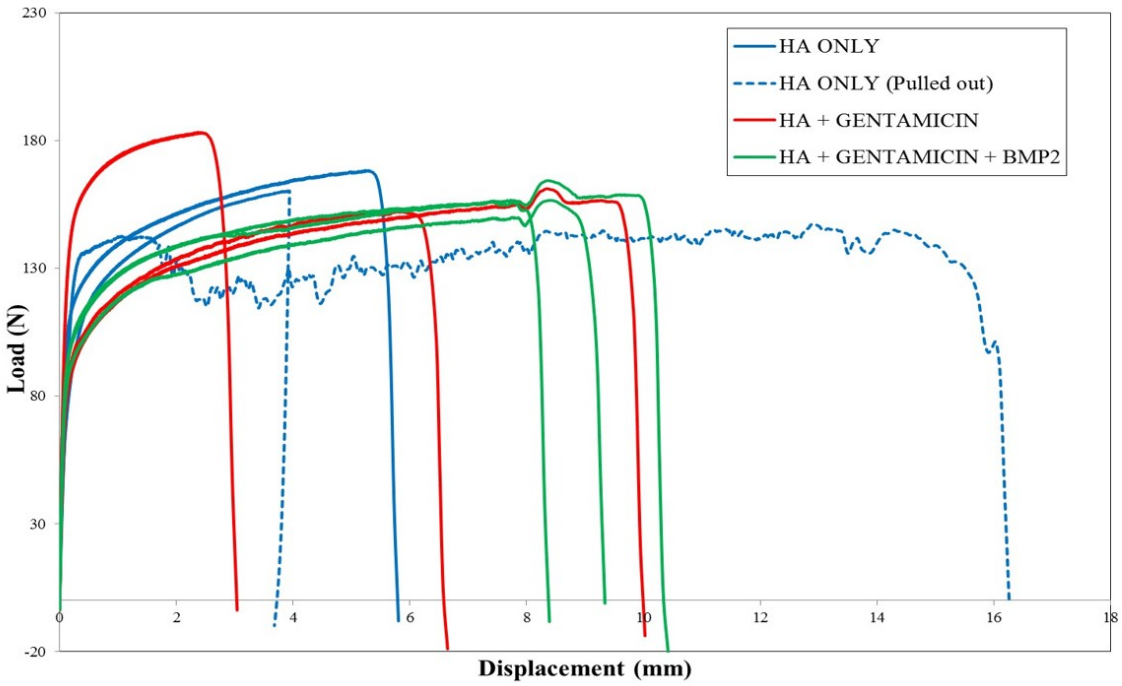

Fig. b. Tensile pull-out, load/displacement graph for hydroxyapatite (HA)-coated titanium wire (HA only), HA-coated titanium wire + sol-gel (SG) and gentamicin (1.25% weight/volume (w/v)) (HA + gentamicin), and HA-coated titanium wire + SG and gentamicin (1.25% w/v) + bone morphogenetic protein-2 (BMP-2) (2 μg ml^{-1}) (HA + gentamicin + BMP2) (n = 3). Mean maximum loads were determined as: HA only = 158.7 N (SD 10.5); HA + SG + gentamicin = 165.6 N (SD 15.9); HA + SG + gentamicin + BMP2 = 159.0 N (SD 4.6).

Video 1. Animated 3D image from micro-CT (μCT) analysis of a transverse section of a rat femur implanted with a gentamicin sol-gel coated titanium rod, at the end of the healing trial. Bone tissue appears red, the titanium implant material is blue, and the coating is green. As detailed in the Methods section above, it was not possible to make a clear distinction between the hydroxyapatite (HA) coating and the sol-gel coating, therefore the

edge of the HA is designated to be equal to the edge of the implant. The dimensions of the implant were 2 mm (length) × 1 mm (diameter).

References

1. **Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A.** The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol*. 1999;37(6):1771-1776.

2. **Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Müller R.** Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res*. 2010;25(7):1468-1486.

NOTE: Please save this file locally before filling in the table, DO NOT work on the file within your internet browser as changes will not be saved. Adobe Acrobat Reader (available free here) is recommended for completion.

The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	See Supp. material: Surgical protocol for in vivo healing non-infection model See Supp. material: Surgical protocol for in vivo healing non-infection model
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	See Supp. material: Surgical protocol for in vivo healing non-infection model See Supp. material: Surgical protocol for in vivo healing non-infection model
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	N/A N/A N/A
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	See Supp. material: Surgical protocol for in vivo healing non-infection model Supp. materia section: Surgical protocol for in vivo healing non-infection model
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Only the field laboratory staff were aware, and the surgical team (Brook and Freeman)
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	Histological evidence of bone growth and contact with implant surface, supported by microCT analysis and 3D reconstruction. n/a
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	Statistical analyses used SPSS statistics software v23 (IBM, Armonk, New York, USA) and GraphPad Prism 6 software (GraphPad, San Diego, California, USA). For pull-out test analysis, the three coating n/a
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Young adult male Wistar rats (weight 230 – 270 g) n/a
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	See Supp. material: Surgical protocol for in vivo healing non-infection model See Supp. material: Surgical protocol for in vivo healing non-infection model Field Laboratories, University of Sheffield This is an established healing defect in the rat femur.
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	See Supp. material: Surgical protocol for in vivo healing non-infection model, and Methods section of main paper. Statistical significance was $p < 0.05$.