

Nanoparticle-based model of anti-inflammatory drug releasing LbL coatings for uncemented prosthesis aseptic loosening prevention

This article was published in the following Dove Press journal:
International Journal of Nanomedicine

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Introduction: The only treatment for aseptic loosening is the replacement of the prosthesis through revision surgery. A preventive approach, achieved through anti-inflammatory drugs released from the device, has shown to be a viable strategy; however, the performance of these devices is not yet satisfactory thus further improvements are necessary.

Methods: We used titanium nanoparticles as a model for implant surfaces and developed a coating containing dexamethasone (DEX) using layer-by-layer deposition.

Results: The amount of deposited drug depended on the number of layers and the release was sustained for months. The efficiency of the released DEX in reducing inflammation markers (tumor necrosis factor alpha and IL-6) produced by human monocytes and macrophages was similar to the pure drug at the same concentration without negative impacts on the viability and morphology of these cells.

Conclusion: These coatings were not inferior to medical grade titanium (the standard material used in uncemented devices) regarding their ability to sustain osteoblasts and fibroblasts growth.

Keywords: titanium, cementless prosthesis, aseptic loosening, dexamethasone

Introduction

Sixty-eight thousand primary hip and 71 thousand knee replacement procedures were performed by the National Health Service in England and Wales in 2017.¹ It is expected that these operations will be performed on an increasing number of patients because of the improvement in life quality they provide and the constantly aging population. The joint replacement device can be fixed to the adjacent bone tissue through two main methods; either using bone cement or cementless fixation which aims to the direct integration of the implant with the host tissue.

Cementless fixation requires a shorter time in the operating room, allows greater preservation of bone stock and provides easier revision;² additionally, uncemented fixation is not affected by any of the possible complications associated with cemented fixation like third body wear and bone cement implantation syndrome.^{2,3} Despite these benefits, instability of uncemented joint replacements was a major drawback that prevented their diffusion.⁴ Modern designs have solved this problem and now these two fixation methods are used in a similar proportion of cases worldwide (although their relative popularity varies in different countries) and have similarly good clinical outcomes.⁴

Aseptic loosening is a severe bone loss (osteolysis) in the tissue surrounding the implant and it is a consequence of a prolonged inflammation;⁵ it is the most likely cause of revision surgery for both hip⁶ and knee⁷ and it is responsible for about

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40% of all revision procedures.⁸ Hence, the development of anti-inflammatory drugs releasing devices has been seen as tool in the fight toward the reduction of arthroplasty failure^{9,10} with the objective of improving on the current NICE guidelines that have set a maximum rate of revision after 10 years for the failure of 10%.¹¹

The direct release of anti-inflammatory drugs from the surface of cementless devices has been suggested as a strategy to reduce the risk of aseptic loosening.^{12,13} The administration of anti-inflammatory drugs does not treat osteolysis but aims at reducing the periprosthetic inflammation that leads to bone loss and consequent aseptic loosening of the device.¹⁴

Dexamethasone (DEX) is a well known anti-inflammatory steroidal drug widely used in clinical practice^{15,16} to reduce inflammation.¹⁷ It is not water soluble, but the esterification with phosphate groups results in dexamethasone phosphate (DEX-P) that is highly water soluble and is the main component of the medical formulations containing DEX.

Layer-by-layer (LbL) is a self-assembly technique employed to produce a coating and it relies on the alternated deposition of positively and negatively charged polymers (polyelectrolytes) through electrostatic attraction.^{18–22} LbL has found medical applications when biocompatible polyelectrolytes are employed. LbL can be also used to release a drug from the coating, replacing one of the polyelectrolytes with the target molecule, whose only requirement is the presence of electrostatic charges. Additionally, to prolong the release of the target molecule, simply more layers are required. Scalability and ease of fabrication of LbL coating are further advantageous characteristics of such technique.^{18–22} The deposition of antibiotics on surfaces using LbL enabled prolonged release of these drugs.^{23,24} Controlled release of DEX from titanium surfaces has not been satisfactorily achieved as the direct conjugation did not sustain release for more than a few days.²⁵ In this work, such steroidal drug was embedded into a dissolvable coating made using alginate and a polymer belonging to the class of poly-beta-amino-esters (PBAEs).²⁶ Titanium nanoparticles were used as a model material for titanium surfaces in light of their high surface/volume ratio and identical chemical structure. The developed material was characterized and the released drug anti-inflammatory activity monitored against human monocytes and macrophages; cytocompatibility was assessed against human osteoblast and fibroblasts. Our research was driven by the following hypothesis: DEX could be embedding in coatings through the use of negatively charged, and water soluble, phosphate form of the

drug (DEX-P), taking advantage of the negative charge exhibited by DEX-P; controlled and prolonged release of DEX would be achieved through LbL; anti-inflammatory activity of DEX would be retained once released from the coatings and no detrimental effects on osteoblast and fibroblast cells would be caused by the coatings activity.

Experimental

Chemicals

Titanium (IV) oxide (Anatase, <25 nm, 99.7%), (3-Aminopropyl) triethoxy-silane (APTS, 99%), PBS tablets, sodium acetate trihydrate ($\geq 99\%$), DEX, DEX-P, 1,6 hexanediol diacrylate and piperazine were purchased from Sigma-Aldrich, UK. HPLC grade acetonitrile, glacial acetic acid and toluene were purchased from Fisher, UK. All other chemicals were reagent grade, stored according to manufacturer's guidelines and used as received.

Polymer synthesis

Amino terminated poly-beta-amino esters, denoted as POLY in the text, were synthesized by mixing 1,6 hexanediol diacrylate and piperazine in DCM with piperazine excess (molar ratio acrylate:amine 1:1.1). The reaction was carried out at 50°C for 48 hrs and the polymers recovered by pouring the reaction mixture in about ten times volumes of diethyl-ether followed by vacuum drying.²⁷

Nanoparticles preparation

Surface functionalization of titanium oxide nanoparticles

Titanium oxide nanoparticles were functionalized with amino groups (Ti-O-NH₂) via silanation in toluene. Briefly, the particles were dispersed in toluene containing APTS for 24 hrs followed by repeated centrifugation/washing.²⁵

LbL coating technique

The amino fictionalized titanium nanoparticles were coated with polyelectrolyte multilayers. The layers consisted of different numbers of a repeating sequence of alginate, POLY, DEX-P and POLY. One sequence containing these four layers was termed quadruple layers, up to ten quadruple layers were coated on the titanium nanoparticles, named as Q_n where n represents the number of quadruple layers. The concentrations used were: 2 mg/mL for sodium alginate and POLY, and 10 mg/mL for DEX-P.²⁷

Nanoparticles surface and material characterization

Transmission electron microscopy – particle size determination

Images of particles were obtained using a Zeiss 902 transmission electron microscope (TEM) operating at a voltage of 80 kV. The aqueous dispersion of the particles was drop-cast onto a carbon-coated copper grid, and grid was dried at room temperature (20°C) before loading into the microscope (direct deposition). The average particle size, size-distribution and morphology analysis of the samples were carried out from at least ten non-overlapping fields of view transmission electron micrographs using ImageJ for Windows (Version 1.50i).

Thermogravimetric assay (TGA)

TGA was performed using a Perkin-Elmer TGA 4000 instrument. Coated nanoparticles were initially weighed and heated from 50°C to 800°C with a heating rate of 10°C/min. Weight loss percentage of each sample at 100°C and 800°C was determined relative to initial weight of the sample. Organic and inorganic content was determined by subtracting the point at initial weight loss (%) up to when the line plateaus (approximately around 800°C).

DEX release quantification

DEX release was measured by dispersing the DEX-coated nanoparticles (10 mg) into 1 mL of a buffer media (acetate buffer pH=5, and phosphate buffer pH=7.3) and incubated at 37°C in Eppendorf.

Daily, the suspension was centrifuged at 4000 g for 10 mins at room temperature; the supernatant was withdrawn for quantification and the medium was replaced with an equal volume of fresh buffer and the nanoparticles resuspended.

The amount of DEX/DEX-P released from the coating layer was determined using reverse-phase High Performance Liquid using an Agilent Technologies® HPLC (1100 series) equipped with a Waters-Spherisorb ODS2 column (Pore size – 80 Å, 5 µm, and packing dimension of 4.6 mm×150 mm). The injection volume was 10 µL. The mobile phase was a mixture of PBS-acetonitrile-glacial acetic acid 70:26:4 at a flow rate of 1 mL/min; a UV detector at 244 nm was employed.

Stock solutions of DEX-P and DEX with a concentration of 1 mg/mL were prepared separately and a series of standards ranging from 0.4 to 250 µg/mL were prepared for calibration.

Cells growth

Human monocytic leukemia cells (THP-1) and human osteoblast cells (Saos-2) were obtained from ATCC and grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin – streptomycin (PS) (Gibco by Life Technologies, USA). Human dermal fibroblast cells were kindly supplied by Prof. Stephens²⁸ from Cardiff University and grown in Minimum Essential Medium Eagle supplemented with 10% heat-inactivated FBS and 1% PS (Gibco by Life Technologies). All types of cells were incubated at 37°C in a humidified 5% CO₂-95% air atmosphere. Media were changed twice per week.

Differentiation of THP-1 monocytes to macrophages

To initiate the differentiation of monocyte-derived macrophages, 30 ng/mL of phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) was added to the RPMI-1640 medium (10% FCS, 1% PS) for 3 days, where human monocytic leukemia cells (THP-1) were grown in suspension. Then, adhered monocyte-derived macrophages were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% PS (Gibco by Life Technologies) at 37°C in a humidified 5% CO₂ atmosphere. Cell medium was changed twice per week.

Exposure of cells to release media containing drug

Media containing DEX released from the LbL-coated nanoparticles was prepared from fresh nanoparticles suspended in buffer pH=7.3 as described in the release experiment protocol; after incubation for 24 hrs at 37°C, the suspension was centrifuged at 4000 g for 10 mins at room temperature. The supernatant was withdrawn and passed through 0.22-µm syringe filter for sterilization; DEX concentration in this solution was 3 mg/L. A solution of DEX-P containing an equivalent concentration of DEX to that in the release buffer from LbL-coated nanoparticles was prepared in buffer pH=7.3 and passed through 0.22 µm syringe filter. These two solutions were added to fresh medium (1:9) to prepare solutions containing 0.3 mg/L of DEX.

Cells were seeded in 96-well plates (Thermo Fisher Scientific, Denmark) at a density of 5×10⁴ cells per well and incubated for 24 hrs at 37°C in a humidified 5% CO₂ atmosphere to allow cell attachment. After removing the media and washing the cells with sterile PBS, cells were incubated for 3 hrs at 37°C in a humidified 5% CO₂ atmosphere with release media from nanoparticles or media containing DEX-P to an equivalent amount of

DEX. The cells incubated in cell medium only with neither 24 hrs solute of nanoparticles nor lipopolysaccharides (LPS) were used as blank control.

1 mg/L of LPS (Sigma-Aldrich) was added to the cells in each well when required. The experiments were performed in triplicate wells to evaluate the cytokines produced by cells and cell mitochondrial activity at two time points, 6 and 24 hrs.

Biological test

MTT

MTT assay was conducted to measure human macrophages (THP-1), human osteoblasts (Saos-2) or fibroblasts mitochondrial activity post-exposure to DEX-coated titanium nanoparticles after 1, 2 and 3 days exposure.²⁴

IL-6 and tumor necrosis factor alpha (TNF α)

The amount of IL-6 and TNF α released by activated and nonactivated THP-1 cells in the media after exposure to LPS and DEX for 6 and 24 hrs was determined using appropriate ELISA kit (Sigma, UK) according to manufacturer's recommendations. The concentration of the inflammation markers was performed by absorbance at 450 nm with (TNF α) or without reference wavelength at 650 nm (IL-6) in a multiwell microplate reader (LT-5000MS ELISA reader, Labtech). Calibration curves were obtained at each measurement to reduce variability between experiments.

Epifluorescent imaging

Cells were fixed with 3.7% (w/v) formaldehyde in PBS at room temperature for 5 mins and, then, permeabilized with 0.1% Triton X-100 for further 5 mins at room temperature.

F-actin cytoskeleton was stained with 50 mg/L of tetramethyl rhodamine B isothiocyanate-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO, USA) for 40 mins at room temperature, followed by incubation with 5 mg/L of trihydrochloride Hoechst 33342 (Thermo Fisher Scientific, Eugene, OR, USA) for 10 mins in the dark to stain cells nuclei. Samples were then washed with PBS, mounted and examined using LSM 880 upright confocal laser scanning microscope with Airyscan (Zeiss, Oberkochen, Germany) with a 63 \times magnification objective. The following settings were employed to excite the dyes and acquire the images: λ_{ex} 540–545 nm and λ_{ex} 340 nm; λ_{em} 570–573 nm and λ_{em} 488 nm for tetramethyl rhodamine B isothiocyanate-conjugated phalloidin and Hoechst, respectively. The obtained

images were processed using ZEN imaging software (Zeiss).²⁹

Statistical analysis

One-way ANOVA was performed to assess the statistical significance of results between groups at a level $p < 0.05$. All data were expressed as mean \pm SD from at least three independent values.

Results

TEM and particles size

Particles appeared cubical and spherical-like shaped (Figure 1A–C) with relatively narrow distributed diameters (Table 1). LbL did not noticeably impact the size of the particles; however, after 10 quadruple layers, some particles agglomeration could be observed.

Thermogravimetric analysis (TGA)

As visible in Figure 1D, the thermograms of all coated nanoparticles showed a plateau from around 650°C that is due to the inorganic fraction (in this work made of TiO₂) of the nanoparticles remaining after the organic components have been burnt.^{30,31} However, the mass loss at this point does not correspond entirely to the amount of deposited polyelectrolyte and drug as the weight loss occurring around 100°C is due to the vaporization of entrapped water not a result of degradation of organic compounds (alginate/POLY/DEX-P); therefore, this mass loss needs offsetting in the calculations.³²

The organic content (Table 2) of uncoated TiO₂ (bare) nanoparticles was the lowest (1.4 \pm 0.2%); while after amino functionalization, the particles presented 4.2 \pm 0.9% of organic fraction. Moreover, in LbL-coated particles, the weight loss at 800°C depended on the number of quadruple layers deposited, from the highest for Q10 (17.5 \pm 1.4%) and the lowest for Q1 (6.6 \pm 0.4). As the organic content is a depended on the film thickness, Q10 was the highest film coating, followed by Q7, Q5, Q3 and Q1.

Drug loading

The amount of DEX loaded on the particles increased with increasing number of quadruples layers deposited (Table 3), starting from 0.021 mg DEX per mg of particles (2.1% w/w) after one quadruple layers to 0.044 mg DEX per mg of particles (4.5% w/w) when 10 quadruples layers were deposited.

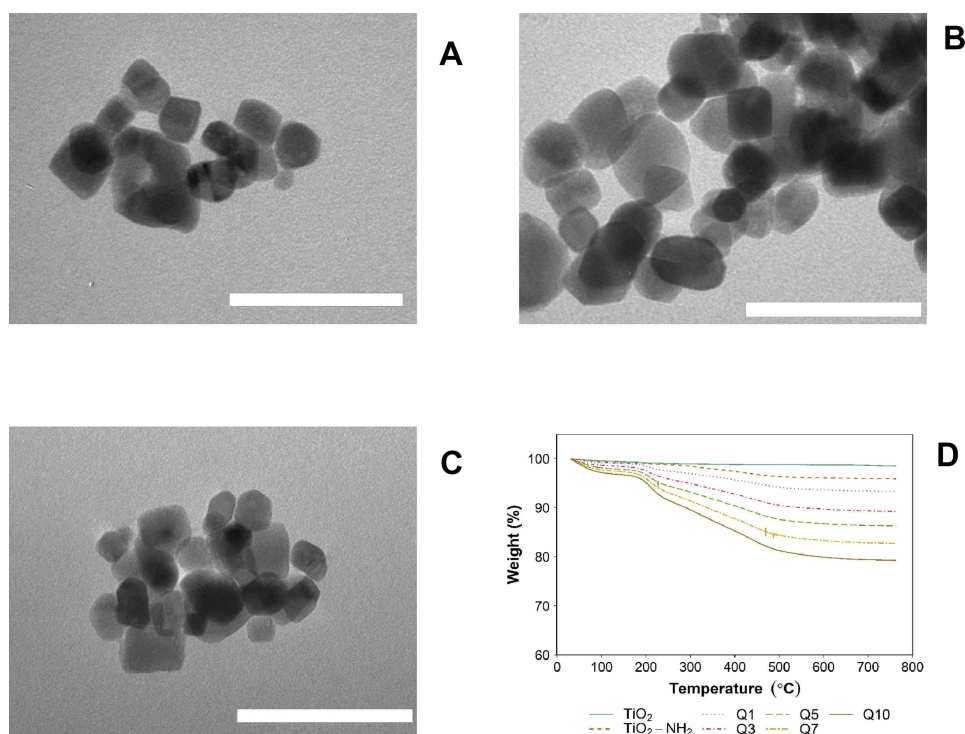


Figure 1 Example of transmission electron microscopy images of (A) bare TiO₂ nanoparticles, (B) amino functionalized (TiO₂-NH₂) nanoparticles and (C) LbL coated (Q10). Bar represents 100 nm. (D) Thermograms of different LbL-DEX-coated TiO₂-NH₂ substrate.

Abbreviations: DEX, dexamethasone; LbL, layer-by-layer.

Table 1 Average diameter size of TiO₂ nanoparticles bare, after functionalization and LbL deposition determined from TEM images

Sample	Average size (nm)±SD
TiO ₂ -particles	34±5
Amino-functionalized particles	34±6
Q10	40±8

DEX release quantification

The amount of DEX released from the LbL-coated TiO₂-NH₂ surfaces monotonically increased with time and overall the entire amount of loaded drug was released at pH=5 regardless of the number of quadruple layers deposited while at pH=7.3 not all the loaded drug was released; the amount of DEX remained entrapped in the coating decreased with increasing number of quadruple layers deposited. DEX was detectable in the release media for longer periods of time with increasing numbers of quadruple layers; moreover, drug release was detectable for up to 20–30 days for both pH conditions (Figure 2).

At pH=7.3, DEX was detected in the release buffer for up to 25 days compared to 30 days for drug release at pH=5; the same pattern (longer release at pH=5 than pH=7.3) was observed for all the particles investigated regardless of number of layers. Furthermore, the total amount of DEX released was

Table 2 Percentage of organic material in multi-layered DEX-LbL-loaded TiO₂-NH₂ surface after addition of various quadruple layers (Q1, Q3, Q5, Q7 and Q10)

Nanoparticles	Organic content (%)
Bare particles	1.4±0.2
TiO ₂ -NH ₂	4.2±0.9
Q1	6.6±0.4
Q3	9.7±0.6
Q5	12.8±0.8
Q7	15.7±0.9
Q10	17.5±1.4

Abbreviations: DEX, dexamethasone; LbL, layer-by-layer.

higher at pH=5 than the one released at pH=7.3 for comparable numbers of quadruple layers.

In-vitro inflammation model: LPS-activated human monocytes and macrophages

Mitochondrial activity

Cellular mitochondrial activity of macrophages after exposure for 6 and 24 hrs to particle release buffer containing 0.3 mg/L of DEX or media containing an equivalent amount of steroidal drug as DEX-P was assessed

Table 3 DEX loading on multi-layered LbL-coated Ti-O-NH₂ nanoparticles after addition of various quadruple layers (Q1, Q3, Q5, Q7 and Q10)

Quadruple layers	DEX loading (mg DEX/mg of NP)
Q1	0.021±0.004
Q3	0.025±0.004
Q5	0.032±0.005
Q7	0.033±0.006
Q10	0.045±0.006

Abbreviation: DEX, dexamethasone.

employing MTT assay and it did not reveal any detrimental effect ($p>0.05$) (Figure 3).

Inflammation markers (IL-6 and TNF α)

Activated human monocytes (macrophages) did not produce either noticeable amount of IL-6 or TNF α when not exposed to LPS. When inflammation was induced, IL-6 reached ~1.2 ng/mL and ~1.4 ng/mL after 6 and 24 hrs of exposure (Figure 4A–B); similarly TNF α concentration was about ~30 and ~33 ng/mL after the same exposure (Figure 4C–D). The addition of DEX-P suppressed IL-6 production

to about a third after both 6 and 24 hrs; TNF α concentration was reduced to half after 6 hrs and about a third after 24 hrs. The use of an equivalent dose of DEX release from LbL coating had similar reduction patterns for both inflammation markers but the efficacy of the release drug was about 20–30% inferior to pure DEX ($p<0.05$).

The concentration of both inflammation markers (Figure 4) released by human monocytes (non-activate) was lower compared to macrophages, but the same pattern was observed with release media containing DEX being capable of reducing the levels of inflammation as pure DEX-P.

Cell morphology

Additionally, cell morphology and cytoskeletal properties were evaluated by staining actin rings using confocal microscopy (Figure 5). The results showed that, after 24 hrs growth in media without LPS and DEX (Figure 5A), the human macrophages were spread over the surface demonstrating a regular morphology. LPS exposure (1 μ g/mL) induced a reorganization in actin distribution of the macrophages resulting in evident difference in cell morphology: cells showed an oval-shaped configuration

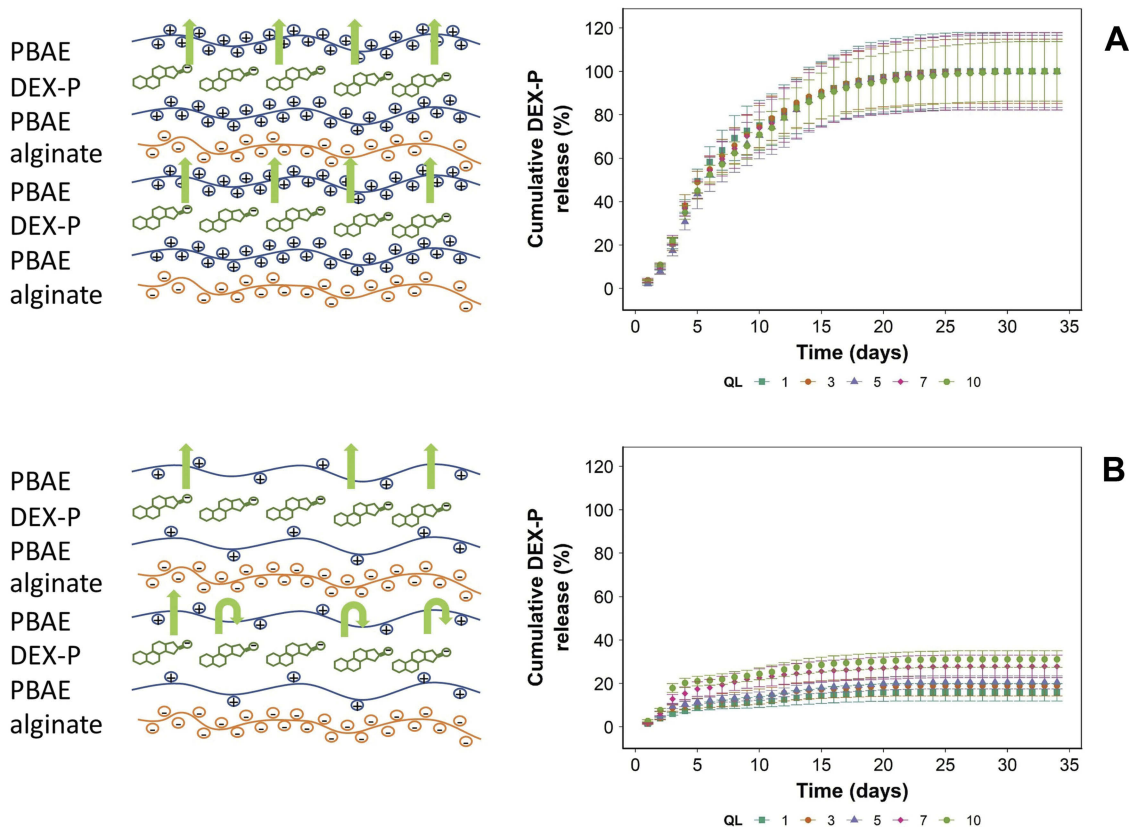


Figure 2 Cumulative release of DEX from LbL-Ti-O-NH₂ surfaces at pH=5 (A) and pH=7.3 (B) for different number of quadruple layers (Q1, Q3, Q5, Q7 and Q10). **Abbreviations:** PBAE, poly-beta-amino-ester; DEX-P, dexamethasone phosphate.

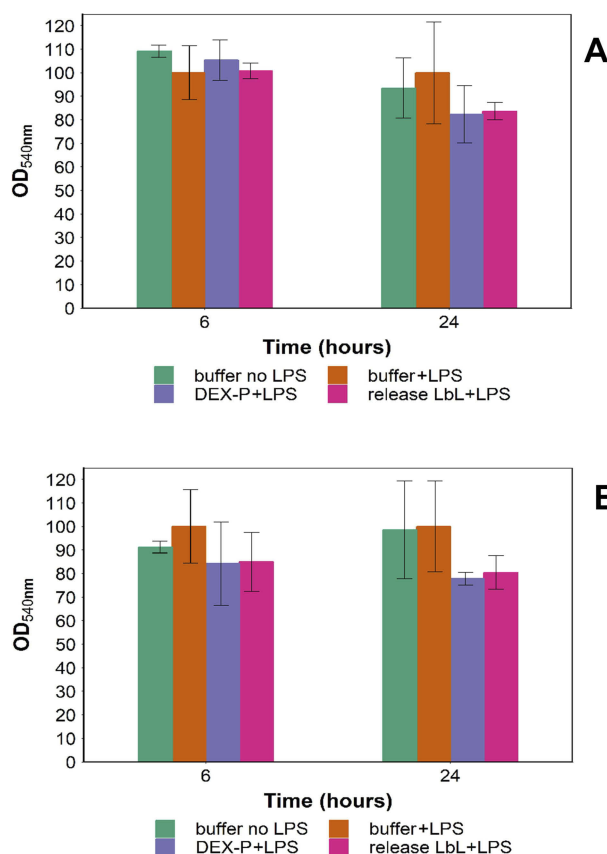


Figure 3 Mitochondrial activity of activated (A) and non-activated (B) THP-1 cells exposed to media containing DEX-P or elutes from DEX released from LbL assembly for 6 and 24 hrs. LPS concentration of 1 $\mu\text{g}/\text{mL}$.

Abbreviations: LPS, lipopolysaccharides; LbL, layer-by-layer; DEX, dexamethasone; DEX-P, dexamethasone phosphate.

(Figure 5B), after addition of DEX the cell size increased remaining oval shape (Figure 5C-D).

Safety assessment for orthopedic application using osteoblasts (Saos-2) and fibroblasts

Mitochondrial activity

The mitochondrial activity of human osteoblasts and fibroblasts exposed to DEX-P or the equivalent dose in release buffers at pH=7.3 increased with exposing time (Figure 6). No difference was observed when comparing control cells to either DEX-P or DEX in release buffers ($p>0.05$) for osteoblasts; while for fibroblasts no difference was detected between DEX-P and DEX released from LbL coatings ($p>0.05$); although both these were lower than the control.

Cell morphology

Osteoblast cells appeared spread with well organized actin filaments regardless of the presence of LPS, DEX-P or DEX from release buffers (Figure 7).

Similarly, the organization of the actin filaments in fibroblast cells was unaffected by the presence of LPS, DEX-P or DEX from release buffers (Figure 8).

Discussion

We successfully developed novel LbL coatings for the release of anti-inflammatory drugs from uncemented joint replacement devices as a preventive approach to aseptic loosening. The properties of these materials were determined to establish, not only the successful preparation of the coatings and the desired drug release profile, but also the active reduction of the inflammatory process and the absence of major cytotoxic effects on the various types of cells the materials would be in contact with.

The size of bare particles determined was in agreement with the manufacturer stated dimensions; amino functionalization did not impact on the nanoparticles size, while the small size increase calculated from TEM images observed after coating with ten quadruple layers is consistent with polyelectrolyte layers thickness under a few nanometers, similar to that reported in other studies.^{27,33} As observed in other studies, polyelectrolyte deposition on nanoparticles surfaces originates

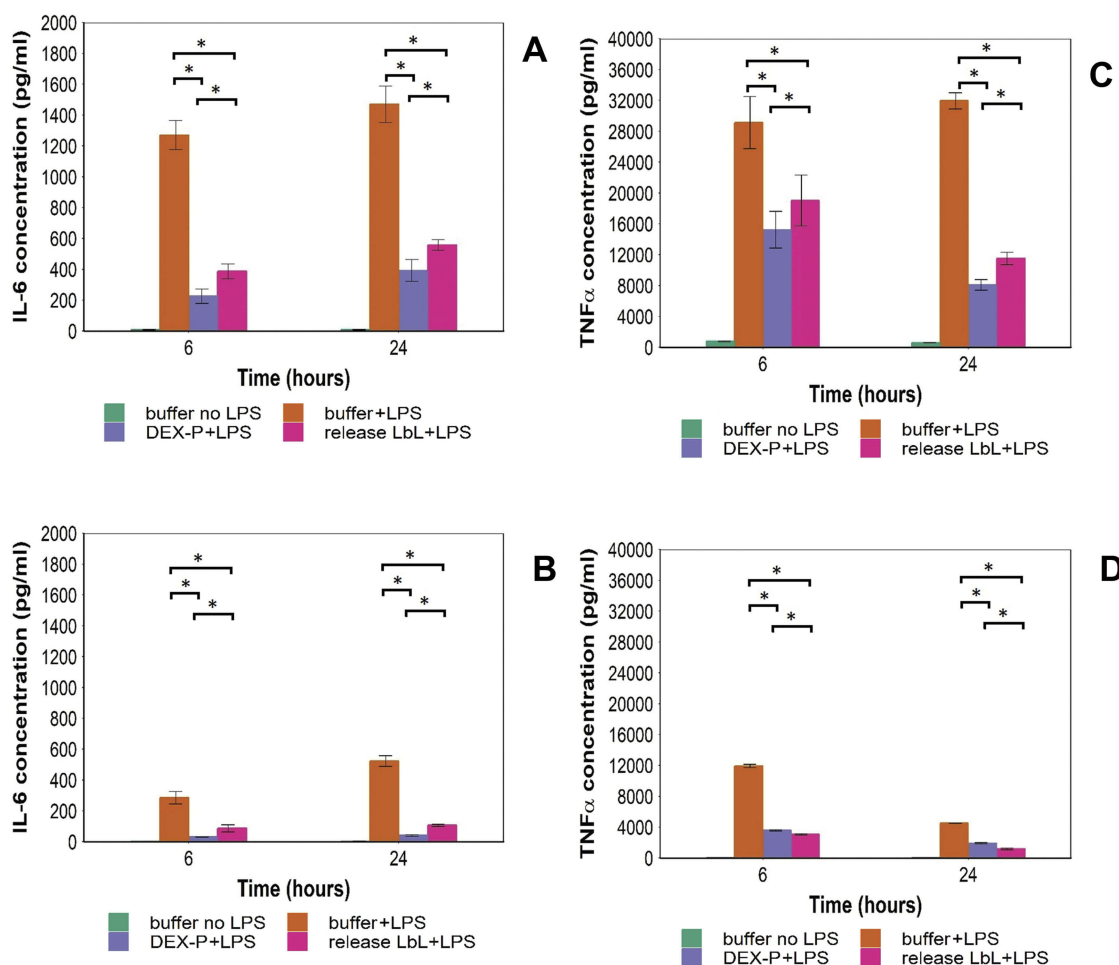


Figure 4 IL-6 expression of activated (A) and non-activated (B); TNF α expression of activated (C) and nonactivated (D) THP-1 cells post-exposure to media containing DEX-P or elutes from DEX released from LbL assembly for 6 and 24 hrs. LPS concentration of 1 μ g/mL was used. (* represents significant differences $p < 0.05$)
Abbreviations: DEX-P, dexamethasone phosphate; LbL, layer-by-layer; DEX, dexamethasone; LPS, lipopolysaccharides; TNF α , tumor necrosis factor alpha.

agglomeration as the polyelectrolytes can deposit simultaneously over more than one particle during LbL assembly.³⁴ These aggregates during subsequent layer deposition behave as one individual particle leading to further agglomeration. However, agglomeration appears to be minimal as LbL protocol was optimized to minimize such occurrence through polyelectrolytes excess.

The amount of organic content on the surface of multi-layered DEX-LbL loaded Ti-O-NH₂ particles, after the deposition of each quadruple layer (alginate, POLY, DEX and alginate) was evaluated by TGA (Figure 1D). Thermogravimetric assay is typically conducted to confirm and quantify the attachment of organic molecules on surfaces either by conjugation³⁵ or LbL.²⁷ The organic fraction for the amino functionalized nanoparticles (Table 2) was the results of the amino silanol conjugating to the titanium surfaces; moreover, the organic fraction increased with growing number of quadruple layers: Q1, Q3, Q5, Q7, Q10 (Table 2). The organic content of the

nanoparticles after each quadruple layer observed in this work was similar to LbL-coated silica nanoparticles containing gentamicin.²⁷ The amount was almost linear with the number of quadruple layers up to Q7 but the rate of organic fraction deposition decreased after this, because, as already seen in TEM images, LbL is progressively hindered by agglomeration and the available surface diminish accordingly.

In-vitro release studies were performed in buffers at two different pH values to simulate different joint conditions: healthy joints (pH=7.3)³⁶ and inflamed joints, which are associated with local acidosis (pH=5).³⁷ Only DEX-P and not DEX was detected in both the release media (pH=5 and pH=7.3). Drug release from LbL constructs is the sum of two simultaneous and independent processes; the progressive detachment of the coating layers (delamination) and the diffusion of the drug through the deposited layers.²¹ The kinetic of the two phenomena depends on the nature of the polyelectrolytes employed and pH;²⁷ these two parameters directly

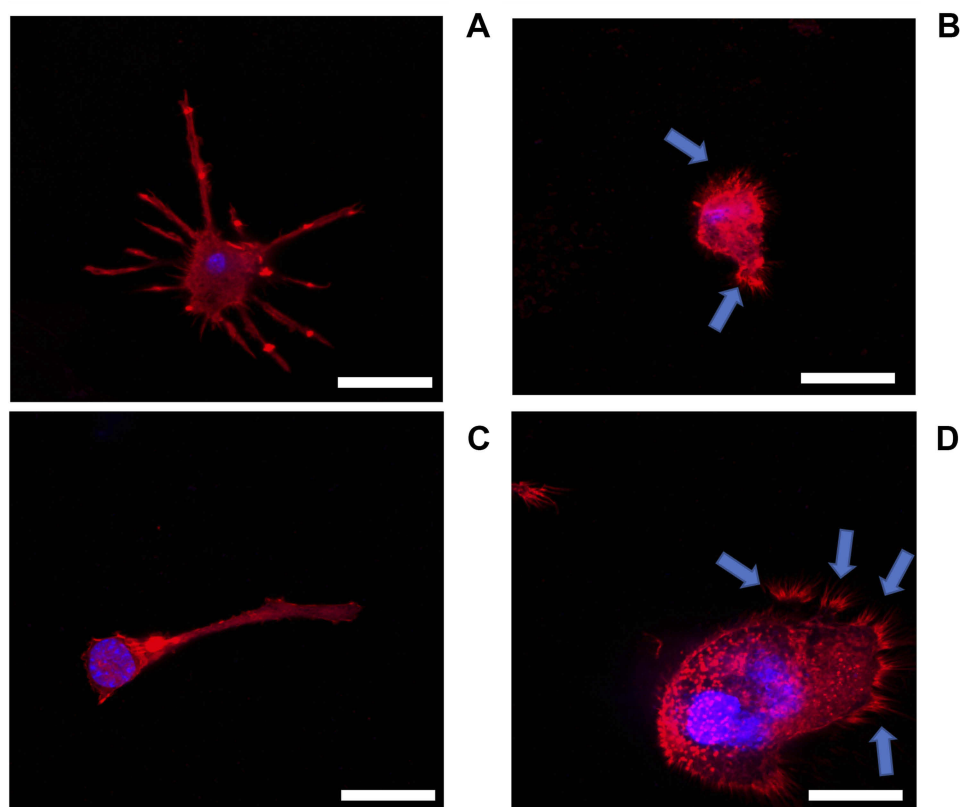


Figure 5 Actin staining epifluorescent images of human macrophages (A) LPS- DEX-; (B) LPS+ DEX-; (C) LPS+ DEX-P and (D) LPS+ DEX from release buffer after 24-hr exposure assessed by confocal microscopy. Actin rings and nuclei of cells were stained with phalloidin-FITC and DAPI, respectively; arrows indicate pseudopods. Bar corresponds to 20 μ m. **Abbreviations:** LPS, lipopolysaccharides; DEX, dexamethasone; DEX-P, dexamethasone phosphate.

influence the hydrolysis kinetic of the polyelectrolytes, that governs the delamination process, and the electrostatic attractions between layers that control the drug diffusion coefficient.^{21,27} Moreover, if diffusion is the predominant mechanism of release the profile exhibits an exponential behavior with the highest drug release at the beginning that gradually drops to zero. On the contrary, pure delamination provides a zero-order kinetic of release (constant rate until the LbL is fully degraded).^{21,27} The release profiles observed in this work (Figure 2) are attributable to release mechanism dominated by diffusion, similar profiles were observed for other LbL coatings prepared with the positive charged antibiotic gentamicin^{18,27} or an osteoinductive protein.³⁸ However, DEX-P exhibited higher release at pH=5 than pH=7.3 while gentamicin exhibited the opposite behavior.²⁷ Higher DEX release kinetic in acidic conditions compared to neutral were also observed³⁹ but determined by the hydrolysis of the ester bond employed to conjugate the steroid. Since only DEX-P was observed in the release buffers, the opposite impact of pH on the release of DEX-P and gentamicin can be assumed to depend on the charge of the two drugs at the pHs tested. POLY is almost completely deprotonated at pH=7.3, with a charge

close to zero, while in mild acidic conditions (pH=5) is fully protonated and positively charged; DEX-P acid groups are deprotonated in both conditions and the drug is negatively charged, analogously alginate is negatively charged in both conditions; both molecules are more negatively charged at pH=7.3 than pH=5. POLY hydrolysis is slow at pH=5,²⁷ more a month to reach a 80% reduction of the polyelectrolyte molecular weight, thus higher delamination does not contribute to the release of DEX-P at pH=5 as it is also revealed by the release profile that is not zero order. Because of the different protonation levels of the LbL components, it can be hypothesized that diffusion of DEX-P deposited in the inner layers of the coatings would be electrostatically hindered at pH=7.3 more than at pH=5. POLY can counteract the negative charges of alginate only in acidic conditions as it is almost neutral at pH=7.3 (Figure 2) hence DEX-P molecules face more electrostatic repulsion from alginate at pH=7.3. This also explains the small impact of the number of deposited quadruple layers on the overall drug release observed at pH=7.3 (Figure 2).

Moreover, drug release had been observed for about 4 weeks and this length of time is compatible with the

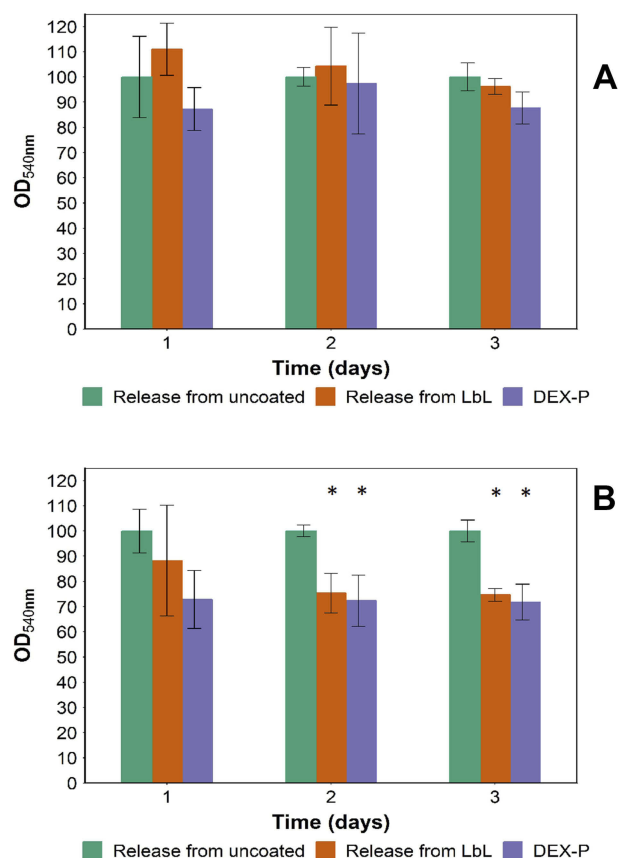


Figure 6 Mitochondrial activity of Saos-2 (A) and fibroblasts (B) cells exposed to media containing DEX-P or elutes from LbL assembly for 1, 2 and 3 days expressed as % of uncoated nanoparticles. (* represents significant differences compared to release from uncoated nanoparticles $p < 0.05$).

Abbreviations: DEX-P, dexamethasone phosphate; LbL, layer-by-layer.

requirements to prevent the acute host inflammatory reaction that occurs immediately after device implantation. This reaction is due to both the injury tissues undergo during surgery to place the device and the immunological response to the material itself.⁴⁰ Acute host inflammatory reaction can result in difficult device integration with compromised functionality and longevity.⁴¹

Inflammation is accompanied by the release of numerous makers in the surrounding tissues (ILs such as IL-6 and TNF α), thus their quantification is employed for diagnostic purposes, including aseptic loosening.⁴²⁻⁴⁴ These inflammation markers induce both osteoclast differentiation and inhibit osteoblast differentiation resulting in an overall bone loss; furthermore, they are also responsible for attracting further macrophages, osteoclasts and lymphocytes to the site aggravating the inflammation.⁴⁵ Both monocytes and macrophages are known to release ILs and TNF α during inflammation⁴⁶ and such human monocytes and activated human monocytes (THP-1)-derived macrophages, as model human

macrophages, were used in this study to test the anti-inflammatory properties of the coatings. LPS concentration of 1 $\mu\text{g}/\text{mL}$ is routinely employed to simulate inflammation in human macrophages^{47,48} while IL-6 and TNF α were chosen to quantify the inflammation process as they are common markers employed in aseptic loosening research.^{49,50} Cells were exposed to LPS and to either DEX-P or the same drug dose using the elutes collected from the DEX release studies at pH=7.3 to confirm that the anti-inflammatory activity of DEX, on inflammation markers such as TNF α and IL-6, was retained when released from the LbL construct. The elutes from the drug release studies performed at pH=5 were not considered due to the low cellular viability they caused as determined in preliminary studies (date not shown). As we have shown (Figure 2) that DEX concentration in the release media varied according to the number of layers deposited onto the Ti-O-NH₂ surfaces, only elutes from DEX released from 10QL assembly were used as maximum concentration of released DEX was obtained.

LbL coatings release not only DEX but also polyelectrolytes or the products of POLY hydrolysis, therefore we determined whether both DEX activity was retained once released, and LbL decomposition products potential toxicity and inflammatory activity. DEX activity once released was marginally reduced compared to the equivalent amount of DEX-P likely as the drug was deposited and not conjugated to the surface, thus avoiding reactions for DEX active groups.

Beside IL-6 and TNF α production (Figure 4), LPS stimulation (1 $\mu\text{g}/\text{mL}$) was shown to induce significant changes on the cytoskeletal properties and morphology of the cells (Figure 5) compared to the untreated cells (control group). Multiple pseudopods with abundant actin filaments were clearly visible only on cells exposed to LPS; these membrane features did not disappear when DEX was added. Similar images were presented by Liu et al⁵¹ and Qin et al.⁴⁹ As monocytes/macrophages phagocytosis is key in their immunological activity, structural changes may have negative impacts on monocytes ability to perform the assigned tasks.⁵²

The development and evaluation of any new biomaterial must comprise not only the assessment of functionality to the desired level, but also the demonstration of no adverse effects caused to tissues and cells that the material will be in contact with. Osteoblasts are the fundamental cells of bones and they are routinely employed in in-vitro testing of novel orthopedic materials,^{53,54} while fibroblasts are present in connective tissues.⁵⁵ Moreover, the impact of the coating on human monocytes (THP-1) and derived macrophages was also determined to exclude drug toxicity toward these cell types.

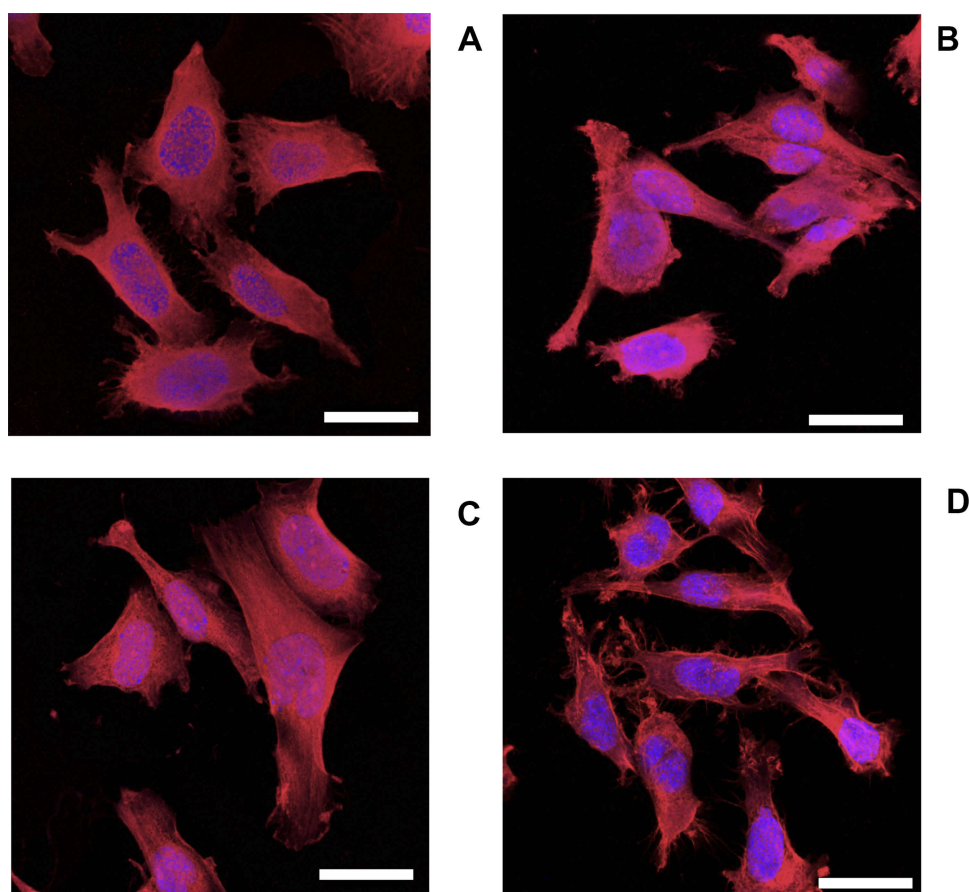


Figure 7 Actin staining epifluorescent images of human osteoblast (Saos-2) (A) no DEX; (B) LPS; (C) LPS+ DEX-P and (D) DEX from release buffer after 24-hr exposure assessed by confocal microscopy. Actin rings and nuclei of cells were stained with phalloidin-FITC and DAPI, respectively. Bar corresponds to 20 μ m. **Abbreviations:** LPS, lipopolysaccharides; DEX, dexamethasone; DEX-P, dexamethasone phosphate.

It was not possible to grow cells directly onto the LbL-coated surfaces as we employed titanium nanoparticles as a model for titanium devices; hence, we tested the response of already established osteoblast cultures to media containing either DEX-P or released DEX to the response to sterile PBS. Moreover, the tests were conducted only from release buffer pH=7 as the acidic buffer (pH=5) exhibited toxic activity.

DEX released from the LbL coating did not negatively impact osteoblasts (Figure 6), fibroblast (Figure 7) and, as such, these materials are not inferior to standard titanium in regards to osteoblasts growth. Furthermore, mitochondrial activity of monocytes and macrophages was not affected by the coatings. Because titanium exhibits sufficient cytocompatibility toward osteoblasts, it was not necessary for the LbL coating to improve such properties and non-inferiority was deemed sufficient. POLY belongs to a class of known biocompatible polyelectrolytes hence the lack of toxic was expected.

In this work, we employed nanoparticles as model for implant surfaces, the next development stage will be the

assessment of whether coatings on larger surfaces (i.e. coupons) would still provide an effective drug dose in light of the lower surface area/unit volume or the amount of quadruple layers necessary to achieve the required drug concentration.

Conclusion

Longevity of joint replacement devices is severely impacted by inflammatory processes that can lead to bone loss and aseptic loosening of the prosthesis. We have successfully controlled the release of DEX (a widely used anti-inflammatory steroidal drug) for about a month. This material did not impact on osteoblasts or fibroblasts proliferation and the anti-inflammatory activity of the realized drug was retained. These materials, therefore, appear a potential tool for reducing the number of revision surgeries necessary when aseptic loosening develops. Uncemented hip replacement devices have a porous or textured surface to allow mechanical interlocking with the bone; hence, during insertion only the outer part of the surface contacts the bone. Because of this, our proposed coating deposited within the porous surface will not be subjected to

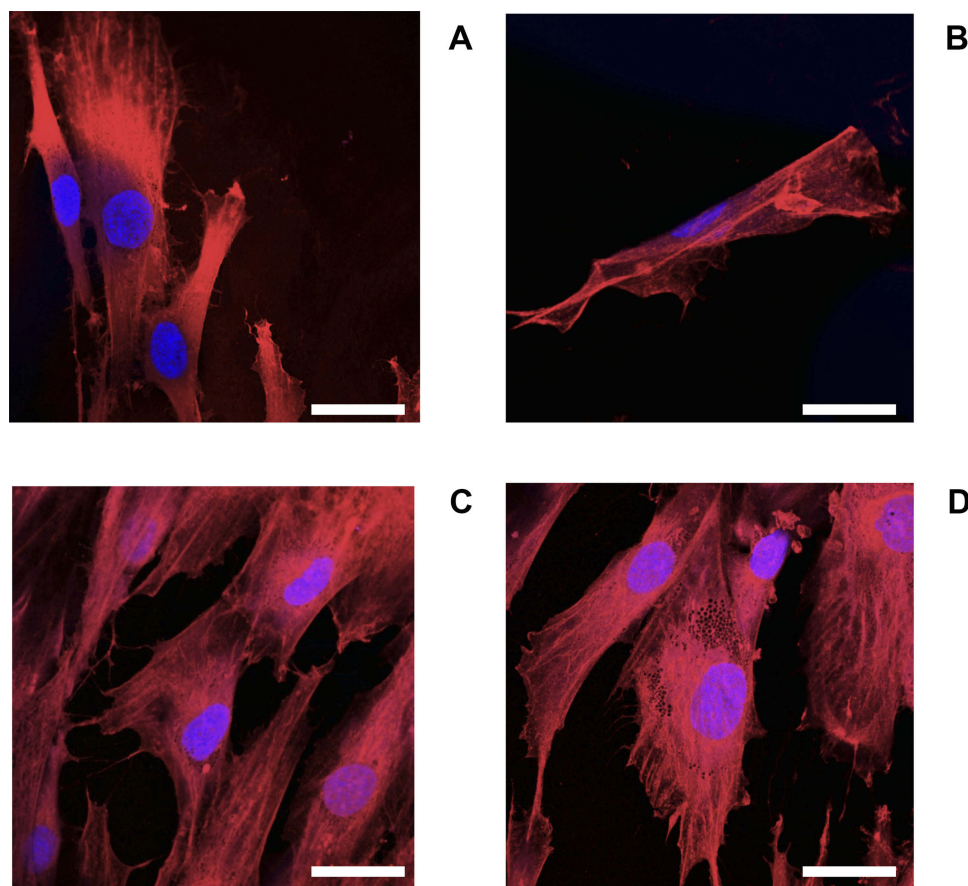


Figure 8 Actin staining epifluorescent images of human fibroblasts (A) no DEX; (B) LPS; (C) LPS+ DEX-P and (D) DEX from release buffer after 24-hr exposure assessed by confocal microscopy. Actin rings and nuclei of cells were stained with phalloidin-FITC and DAPI, respectively. Bar corresponds to 20 μ m.

Abbreviations: LPS, lipopolysaccharides; DEX, dexamethasone; DEX-P, dexamethasone phosphate.

friction and will remain unaffected by the handling and mechanical actions associated with the surgery.

Acknowledgments

This work was supported by funding from the Princess Nourah Bint Abdulrahman University, Saudi Arabia, and Arthritis Research UK (ARUK: 18461).

Disclosure

The authors have no conflicts of interest to declare.

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