Impact of collagen/heparin multi-layers for regulating bone cellular functions

*Ana M. Ferreira *†,‡, Piergiorgio Gentile †, Sotiria Toumpaniari†, Gianluca Ciardelli ‡, and Mark A. Birch* ∓*

† Mechanical and Systems Engineering, Newcastle University, Newcastle upon Tyne, United Kingdom

‡ Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Turin, Italy

∓ University of Cambridge, Division of Trauma & Orthopaedic Surgery, United Kingdom

ABSTRACT. Bone cell interaction with extracellular matrix (ECM) microenvironment is of critical importance when engineering surface interfaces for bone regeneration. In this work layer-by-layer films of type I collagen (coll), the major constituent of bone ECM, and heparin (hep), a glycosaminoglycan, were assembled on poly(L-lactide acid) (PLLA) substrates to evaluate the impact of the biomacromolecular coating on cell activity. The surface modification of PLLA demonstrated that the hep/coll multi-layer is stable after 10 bilayers (confirmed by contact angle, infrared spectroscopy and morphological analysis). This simple approach provided novel information on the effect of heparin on type I collagen hierarchical organisation and subsequent cell response of osteoblast-like (MC3T3-E1) and human bone marrow-derived mesenchymal stem cells (hMSCs). Interestingly, the number of deposited heparin layers (1 or 10) appeared to

play an important role in the self-assembly of collagen into fibrils, stabilising the fibrous collagen layer, and potentially impacting on hMSCs activity.

KEYWORDS. Collagen, heparin, layer-by-layer, mesenchymal stem cells, surface modification

INTRODUCTION

The use of materials that are both biodegradable and biocompatible for the manufacture of biomedical implants for bone regeneration has increased. Poly (lactic acid) is one of the most used polymers in the field of orthopedics due to its biocompatibility, biodegradability, thermoplastic processability and mechanical properties[1]. This material has been used for developing sutures, screws and fixation plates in bone fractures, constructs for bone and cartilage repair, drug release systems, and devices for ligament/tendon healing [2]. With advances in tissue engineering, there is the opportunity to promote bone regeneration rather than provide a mere structural support. An optimal bone graft material should induce osteogenesis and therefore promote appropriate osseointegration. By incorporating specific structural components such as collagen, and GAGs to mimick the native extracellular matrix (ECM) microenviroment at the surface of the implant it is possible to modulate specific cellular interactions and influence localized tissue response.

Type I collagen has previously been demonstrated to accelerate osteoblast differentiation and matrix mineralisation *in vitro* [3]; however a critical level of collagen (~2.4 mg/ml) is required to achieve this effect [4]. Glycosaminoglycan (GAG) chains on ECM proteoglycans are able to bind different growth factors and maintain the required ECM-space for bone development [5]. Heparin is a highly sulphated GAG capable of storing and releasing growth factors [6]. Several proteins

contain heparin-binding domains, resulting in a strong interaction between this polysaccharide and the ECM components involved in cell adhesion [7], proliferation and osteogenic differentiation [8]. Combining collagen and heparin is therefore a system based on natural compounds with unique biological properties, that are Food and Drug Administration (FDA) approved, and can be easily applied to clinical applications. Previous studies on the interaction of collagen and heparin have demonstrated that heparin has a direct impact on the kinetics and morphology of reconstituted collagen type I fibrils [9-10]. Collagen and heparin has been reported as a coating of tricalciumphosphate/hydroxyapatite (TCP/HA) granules for long term delivery of BMP-2. A local and sustained delivery system for BMP-2 after 21 days was obtained, suggesting this collagen/heparin system as a potential BMP-2 carrier to enhance bone regeneration in large bone defects [11]. The response of mesenchymal stem cells (MSC's) to the combination of collagen and heparin, has been previously studied on aligned collagen type I matrices using a microfluidic set-up to then incorporate the heparin. The study has reported that the extent of osteogenic differentiation was not significantly affected by aligned and heparin-functionalised substrates [12]. In this context the lack of effect of collagen and heparin on osteogenesis and MSC's differentiation could be related to the very specific collagen/heparin organisation, topography, and chemical composition.

To overcome these limitations, the layer-by-layer (LbL) assembly method possesses advantages for recreating native ECM microenviroments for bone tissue regeneration by allowing the self-assembly of highly organized 3D complex structures *in vitro* [13-14]. Due to its versatility and simplicity for incorporating high levels of different types of biomolecules with a fine control over multilayer structures, by alternating adsorption of oppositely charged polyelectrolytes, LbL assembly provides a rational method towards the control of cell behaviour at the implant

interface. Previous studies using LbL assembly of collagen and heparin have been performed on titanium [15-18], porous hydroxyapatite[19], ePTFE[20] to study blood compatibility, vascularisation and angiogenesis. Although, the collagen and heparin system has been studied mostly as a carrier for drug delivery or system to promote vascularisation, the polyelectrolyte multilayers of collagen and heparin on a PLLA biodegradable polymer and its effect on bone cells and human mesenchymal stem cells has not previously been investigated.

In this work, we optimised and confirmed successful LbL heparin/collagen assembly on PLLA using water contact angle, attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and X-ray Photon Spectroscopy (XPS) to evaluate the effect of the heparin and collagen functionalisation (varying the numbers of the final layers) on the physical-chemical characteristics. Furthermore, the selected collagen/heparin multilayers were further investigated *in vitro* by culturing osteoblast-like (MC3T3-E1) and human bone marrow-derived mesenchymal stem cells (hMSCs) at short and mid-term, respectively. Results reported in this work are relevant for understanding the effect of heparin (GAGs) on collagen properties as main structural protein in our body and their impact on MSC activity and osteogenesis when engineering construct interfaces to enhance biocompatibility, modulate specific cell behaviour, facilitating differentiation and matrix mineralisation.

MATERIALS AND METHODS

Materials: Type I collagen was isolated and purified form rabbit bone after pepsin digestion as described by Ferreira et al. [21]. Sodium heparin was purchased from Polysciences Inc. (Italy, Mn: 100M USP Units). PLLA 3051D was purchased from PuracBiochem (Italy, Mn of 100,000 Da). Bi-distilled water was used in all experiments. Conventional circular borosilicate glasses

coverslips for microscopy with 13 mm diameter (VWR, UK) were used for the preparation of the nano-coated PLLA films via LbL assembly. Cleaned borosilicate slides were used as control. Dulbecco's modified Eagle's medium (DMEM) from Sigma-Aldrich (UK) was used containing 1000 mg glucose/L pyridoxine HCl and $NaHCO_3$ and, supplemented with 10% of heat inactivated fetal bovine serum (FBS; from LonzaTM, UK), 2mM L-glutamine and 100 units/ml penicillin/ 100 µg/ml streptomycin (Gibco, Life technologies Ltd. All media was kept refrigerated at 4°C and pre-warmed at 37°C before use.

Preparation of PLLA films by spin coating. The borosilicate glasses were cleaned using "Piranha cleaning solution" (mixing 1:1 v/v ratio of sulphuric acid and hydrogen peroxide - H_2SO_4 and H_2O_2, Sigma-Aldrich, Italy) in order to remove any impurities or particles adhered to the surface that could interfere with the further chemical functionalisation. After one hour, glasses were rinsed three times with distilled water plus once with 70% ethanol solution (Sigma-Aldrich, Italy), dried with nitrogen gas and stored under vacuum. Silanisation treatment was performed on cleaned glasses to promote the chemical interaction between polymer and glass substrates and prevent the peeling-off of the polymeric film along the experiment. In details, cleaned glass substrates were immersed into 2% solution of 3-aminopropyltriethoxysilane in diethylene dioxide (1,4-dioxane; Sigma-Aldrich, Italy) for 3 hours at room temperature, protected from light and water. Silanised glasses were rinsed three times with 1,4-dioxane, followed by 20 min of ultra-sonication in fresh 1,4-dioxane and dried with nitrogen gas. A puddle of 35 µl PLLA 5% (v/w) solution in 1,4-dioxane was deposited onto the centre of the glass substrate, and spun at 1000 rpm for 3 s followed by 10 s at 7000 rpm using a spin-coater instrument (Bench Top Spin Coater, MTI tech, LTD, UK). Glasses were spin-coated twice with PLLA solution to increase the polymer film thickness and heated at 70°C (over the PLLA glass

transition, approx. 65°C) for 45 min in order to facilitate the reaction between the amino groups present on silanised glass and the ester-groups of PLLA polymer, to ensure the evaporation of remaining solvent and to anneal any orientation or mechanical stresses induced by the spin coating process.

Heparin and collagen Layer-by-Layer assembly. PLLA films were aminolysed by treating them during 14 min into 0.06 g/ml solution of 1,6-hexanediamine/2-propanol (Sigma-Aldrich, Italy) at room temperature (25°C). Aminolysed films were washed with a large quantity of water and dried under reduced pressure at 30°C [22-23]. The amount of amino groups present on aminolysed PLLA surfaces was determined by the Acid Orange II (AOII, Sigma-Aldrich, Italy), also known as Acid Orange 7. This colorimetric method is based on the principle that positively charged amino groups on material surfaces can combine by an ion exchange mechanism with negatively charged dye under acidic conditions [24]. Briefly, films of 1 cm2 were immersed in 1 ml of an aqueous solution of 500 µmol/l Acid Orange II at pH 3 (diluted HCl) and incubated under agitation at room temperature for 24 h to carry out the protonation of the amines. Films were washed with acid water at pH 3 (diluted HCl). The bound dye was released in 1 ml of distilled water at pH 12 (diluted NaOH) after 15 min of shaking at room temperature and measured spectrometrically at a wavelength of 492 nm (UV-Vis Spectra PerkinElmer, Lambda 25). From a standard curve, the concentration of amino groups was determined using the assumption that one amino group ($–NH_2$) is complexed by one equivalent of Acid Orange II. All measurements were performed in triplicate.

Positively charged PLLA surfaces were coated with multiple alternating layers of collagen and heparin as shown in figure 1. The LbL deposition was performed by depositing 50 µl of biopolymer solution onto the aminolysed PLLA surface for 10 min, starting with the heparin

(hep) as first layer, followed by a washing step with distilled water for 10 min to remove unbound molecules of heparin. Collagen (coll) solution (50 µl) was deposited during 10 min, followed by washing with distilled water; repeating the entire cycle up to ten times. Biopolymer solutions - heparin and collagen - were prepared with concentration of 1 mg/ml and pH adjusted to 5. Distilled water was used to prepare all solutions, including the washing baths.

The final multilayers were coded with the initial letter of the last polyelectrolytes coated (C for collagen and H for heparin), followed by the number of bilayer created.

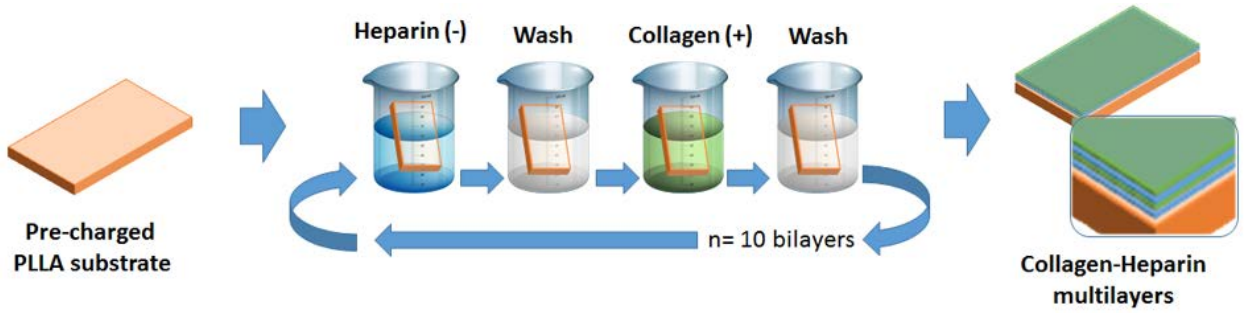

Figure 1. Illustration of Layer-by-Layer process with collagen and heparin onto poly-lactic acid polymeric substrates.

Physico-chemical analysis of multi-layered samples. Surface morphology of hep/coll coatings and controls were evaluated by Scanning Electron Microscopy technique (SEM LEO1430VP). SEM micrographs were taken at varying magnification, using working distance (WD) of 10mm and an electron high tension of 20kV (EHT). Prior to the SEM analysis, samples were coated with a conductive thin gold layer.

Surface hydrophilicity of controls and heparin/collagen layers was evaluated by Static Water Contact Angle. The measurements were performed at room temperature using a Contact Angle CAM 200 KSV Instrument, equipped with Tetha software using the sessile drop method, dispensing 3 µl water droplet (HPLC grade) directly on the sample surface by a motor driven

syringe. Contact angle measurements were analysed using automatic software and presented as the average of three measurements from three different points on each surface.

Chemical properties of deposited heparin and collagen layers were evaluated by mid-infrared spectroscopy. FTIR spectra of 36 scans at 4 cm^{-1} resolutions were obtained by a Frontier PerkinElmer FT-IR spectrophotometer with a universal attenuated total reflectance (ATR) module in Germanium crystal.

Surface composition was determined by X-Ray Photoelectron Spectroscopy (XPS; Theta Probe, Thermo Scientific, East Grinstead, UK), which uses a micro- focused AlKa X-ray source (1486.6 eV), operated with a 400 µm spot size (100 W power). Survey spectra were collected at a pass energy of 200 eV, a step size of 1 eV and a dwell time of 50 ms, with the spectrometer operated in standard (not angle-resolved) lens mode. High resolution regional spectra were collected using a pass energy of 40 eV, a step size of 0.1 eV and a dwell time of 200 ms. High resolution spectra envelopes were obtained by curve fitting synthetic peak components using the software CasaXPS.

Quantification of the biomolecules assembled within the films. Taylor`s Blue colorimetric method was used to quantify heparin content. Taylor's Blue solution was prepared by dissolving 3.04 g glycine (Sigma-Aldrich, Italy), 2.37 g NaCl (Sigma-Aldrich, Italy) and 16 mg of 1,9-dimethylmethylene blue dye (Sigma-Aldrich, Italy) in 95 mL of 0.1 M HCl and completed with deionized water to make the solution up to 1 liter. Films were cut into square samples with a surface of 1 cm^2 and incubated in 1 ml of Taylor's Blue dye reagent in capped tubes for 30 min. Samples were washed with deionised water and transferred to new tubes. Then, 1 ml of dissociating agent (3M Guanidine hydrochloride - GuHCl (Sigma-Aldrich, Italy)) in Phosphate Buffer Saline (PBS, Sigma-Aldrich, Italy) at pH 7.5) was added to each sample and shaken in a

vortex mixer for 10 min to facilitate the release of the bound dye 1,9-dimethylmethylene blue dye molecules into solution, turning the coloration of dissociating reagent from crystalline to blue. Each sample solution was transferred to disposables cuvettes and the absorbance was measured at 653 nm using an optical spectrophotometer (Perkin Elmer Instrument Lambda 40). Measured absorbance was assessed against dissociation reagent alone for the blank, calibration curve standards and test samples. Heparin density was calculated through a calibration curve previously built at different known heparin concentrations (0-5 µg/mL). All the measurements were performed in triplicate.

The amount of collagen present in the multi-layered PLLA films was quantitatively determined using Sirius red-based colorimetric method. Films were cut into square samples with a surface of 1 cm^2 and incubated into 1 ml of Sirius red dye reagent into a cap tube for 30 min in order to form a collagen-dye complex. Sirius red solution was prepared by dissolving 0.5 g of Direct Red 80 (Sigma Aldrich, Italy) into 500 ml of saturated aqueous solution of picric acid (1.3% in water). Thereafter, the dye solution was drained and the stained samples were washed twice with iced-cold acidified water (containing 0.5% acetic acid and sodium chloride) in order to remove the unbound dye. The washing solution was removed as well as any fluid from the lip of the eppendorf tubes using cotton wool buds. The bound dye was dissolved by adding 1 ml of the alkali reagent (0.5 M sodium hydroxide) to the samples and mixed for 10 min. The collagen bound-dye was transferred into disposables cuvettes and optical density was measured at 560 nm with UV-Vis scanning spectrophotometer (PerkinElmer, Lambda 40). The density of collagen bound to PLLA films was calculated using a calibration curve prepared with known collagen concentrations. All the measurements were performed in triplicate.

Short term in vitro evaluation of multi-layered films with collagen and heparin using MC3T3 cells. Short-term in vitro study (up to 3 days) was performed using the pre-osteoblastic cell line, MC3T3-E1 to evaluate the influence of hep/coll layer-by-layer films on cell morphology and viability. Prior to seeding the cells on surfaces, samples were sterilised with UV light (Polaris UltravioletTM germicide lamp) at 208 nm for 30 min.

MC3T3-E1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco), 2 mML-glutamine (L-G), 100 units/ml penicillin and 100 µg/ml streptomycin (P/S). One millilitre of MC3T3-E1 cells suspension containing 10,000 cells was dispensed onto each samples films (Ø= 13mm). Cells were incubated on the multi-layered substrates placed in 24-well tissue culture plates (Lonza, UK) at 37 °C in an atmosphere containing 5% CO_2 for up to 7 days. All experiments were repeated three times.

Cell morphology was characterised by fluorescent microscopy. For this purpose, culture media was removed from each well and samples were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS), Lonza (UK). Cells adhered to the sample surface were fixed in 4% (w/v) paraformaldehyde (Sigma Aldrich, UK) in PBS preheated at 37 °C for 20 min. Subsequently, fixative solution was drained and samples were washed three times with DPBS/0.1% (v/v) Tween 20. Samples were blocked in 3% (v/v) goat serum (Sigma Aldrich, UK) in DPBS/0.1% Tween 20 for 30 min and then incubated with 200µL of Rhodamine phalloidin (Sigma Aldrich, UK) in DPBS/0.1% Tween 20 [1:1000] for 20 mins. Samples were then washed three times with DPBS/0.1% (v/v) Tween 20 and mounted onto microscope glass slides (VWR, UK). Cell nuclei were visualised with DAPI (Sigma Aldrich, UK) included in the anti-fade mounting medium (Vector Labs Inc). Samples were imaged on a fluorescent microscope DMLB-

Leica Fluorescent light Microscope combined with Camera-Advanced SPOT. The cell and nucleus shape analyses were performed manually by defining the regions of interest using Image J software.

Cell viability was evaluated by using the MTT assay (Sigma Aldrich, UK). For this purpose, the culture media was removed and 200 µl of 1 mg/ml MTT in DMEM (phenol red and serum free) was added to each well. Samples treated with MTT reagent were incubated for 4 hours at 37°C. Then, media was carefully removed and 100 µl of isopropanol was added to each well to solubilise the tretrazolium crystals. The multi-well plate was covered with tinfoil and agitated on an orbital shaker for 30 min. Solubilised formazan (100 µl) was transferred to a 96-multiwell plate and the light absorbance read at λ=560nm using a Sunrise Elisa plate reader (XFLUOR4 V4.51).

In vitro study of coll/heparin multi-layered films using human bone marrow-derived mesenchymal stem cells (hMSCs). In vitro cellular studies of layer-by-layer films of heparin and collagen were evaluated in short-term (up to 7 days) and long-term (up to 28 days) experiments using hMSCs isolated from a 21 years old female (Lonza Inc.,USA). hMSCs were seeded in basal media (DMEM-low glucose without glutamine, 10% heat-inactivated fetal bovine serum, 2 mM L-Glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin) and incubated in 5% CO2 humidified atmosphere at 37°C. Basic fibroblastic-growth factor (b-FGF) at a concentration of 10 ng/ml was added in order to stimulate cell proliferation. Cells at passage 5 were used in this experiment. Samples (in quadruplicates) were placed in a 24-well tissue culture plate (Lonza, UK) and one millilitre (1ml) of hMSCs suspension containing 20,000 cells was added to each sample: PLLA substrates, 1 layer of heparin (H1), 1 layer of collagen (C1) and 10-layers of collagen (C10).

Metabolic activity on hep/coll multilayers was measured by MTT based assay at 1, 3, 7, 14 and 28 days, as described previously. The activity of alkaline phosphatase (ALP) was assessed using the p-nitrophenol assay after 7, 14, and 28 days of culture. Nitrophenyl phosphate (pNPP; Sigma-Aldrich, UK), which is virtually coluorless shifts to a yellow end-product, p-nitrophenol, when hydrolyzed by alkaline phosphatase. The culture media was removed from cell cultures and samples were rinsed three times with DPBS. Samples were fixed by adding 300µL of 4% of parafolmadehyde fixative solution preheated at 37°C to each well and left for 10 min. Fixative solution was drained and samples were washed twice with DPBS, followed by one last wash with 0.1M Tris, pH 8.3 to alkalinise the samples. Fixed cells were incubated with 500ul of pNPP liquid substrate system at 37°C for 30 min, until yellow colour developed. Subsequently, 100µl of each sample was transferred in triplicate into a 96-well plate and ALP values were read at 405 nm in a Sunrise plate reader (Tecan). A standard curve for absorbance was prepared by serial dilutions of 50µmol/L p-NP p-nitrophenol solution in 0.1M Tris (pH 8.3), transferring 100ul of standards and blanks to a 96-well plate. ALP concentrations were read off from the standard curve. ALP was normalised with the total number of cells adhered to the samples surface determined through the Crystal violet assay, and time of ALP incubation (30 min). Cells were fixed by adding 300µL of 4% (w/v) of paraformadehyde and incubated with 200ul of 0.1% (w/v) pre-filtered Crystal Violet solution for 10 min at room temperature. The stained cells were then washed with water three times, air dried and crystal violet solubilised with 100ul of 95% of ethanol for 15 min at room temperature. Solubilised crystal violet was mixed and transferred to a 96 multi-well and light absorbance measured at λ= 570nm using a Sunrise Elisa plate reader (XFLUOR4 V4.51).

Histochemical staining was performed to assess attached cells population expressing ALP enzyme and identify mineral deposits. Briefly, Naphthol solution was prepared by adding 5 mg of Naphthol AS MX-PO4 (Sigma) to 200µl of DMF (N,N-Dimethylformamine, Fisher Scientific) and 30 mg of Fast Blue RR (Sigma) to 25 ml Tris-HCl (MW. 157.6), 0.2M, pH 8.3. Both solutions were mixed and filtered, and distilled water was added to complete the substrate solution. After the washing time, water was removed and samples were incubated with 500ul of Naphthol substrate during 45min at room temperature. Samples were rinsed three times with ultrapure water and stored in water for 5 min. Mineral deposits was determined by Alizarin Red staining. Wash water was carefully aspirated from wells and samples were incubated with 300 µl of 2% (w/v) Alizarin Red solution, pH 4.2 at room temperature for 30 min. Alizarin Red Solution was then removed and samples were washed several times with distilled water. ALP positive areas appear purple, while calcium deposits were stained orange/red by the Alizarin Red solution and visualised by bright field microscopy (DMLB-Leica Fluorescent light Microscope combined with Camera-Advanced SPOT).

Statistical analysis. The statistical analysis in terms of significant difference of collagen biomolecules quantification and cell adhesion and ALP expression was performed for n= 3 samples by ANOVA (one-way analysis of variance) using Origin-7 software. The post-hoc multiple comparisons between the independent groups were evaluated using the Tukey's test.

RESULTS AND DISCUSSION

To allow LBL assembly on the polymer, PLLA films were activated through an aminolysis process in order to positively charge the surface, and favour the absorption and electrostatic interaction with first polyelectrolyte layer. Primary amines introduced by aminolysis treatment

onto PLLA substrates were quantitatively assessed by the acid orange II assay. The density of NH_2 groups on the substrates was determined to be 1.6 ± 0.5 μg/cm^2. Surface hydrophilicity was slightly increased after the aminolysis treatment of the surfaces, with lower water contact angles (about 75 ± 3°) compared to untreated PLLA surface values of 79 ± 3° (Figure 2A).

Heparin and type I collagen were used as bioactive polyions for LbL assembly in order to create a surface that mimics the characteristics of the ECM, as reported also in literature for other applications [20]. The wettability of surfaces coated with hep/coll multilayers was evaluated by water contact angle (Figure 2A). Water contact angles were measured for controls (PLLA and aminolysed surfaces) and for 1, 5, 10 and 20 hep/coll multilayers. No-major changes in terms of surface wettability were observed after the deposition of one bi-layer of hep/coll in comparison with the control (contact angle values close to 80°). However, the deposition of five bilayers of hep/coll enhanced significantly the hydrophilicity of the polymeric surface, resulting in contact angle values in the range of 60° to 70°. Contact angle values were similar (about 62°) on surfaces coated with 10 and 15-bilayers of hep/coll. However, the surface hydrophilicity was significantly reduced when the number of deposited hep/coll films was increased up to 20-layers of collagen. This result suggests that the surface has become saturated, decreasing the electrostatic stability between the multilayers and preventing deposition of additional coating.

The chemical surface properties of 1, 5, 10, 15 and 20 multilayers of heparin and collagen were evaluated by FTIR-ATR technique (Figure 2B)

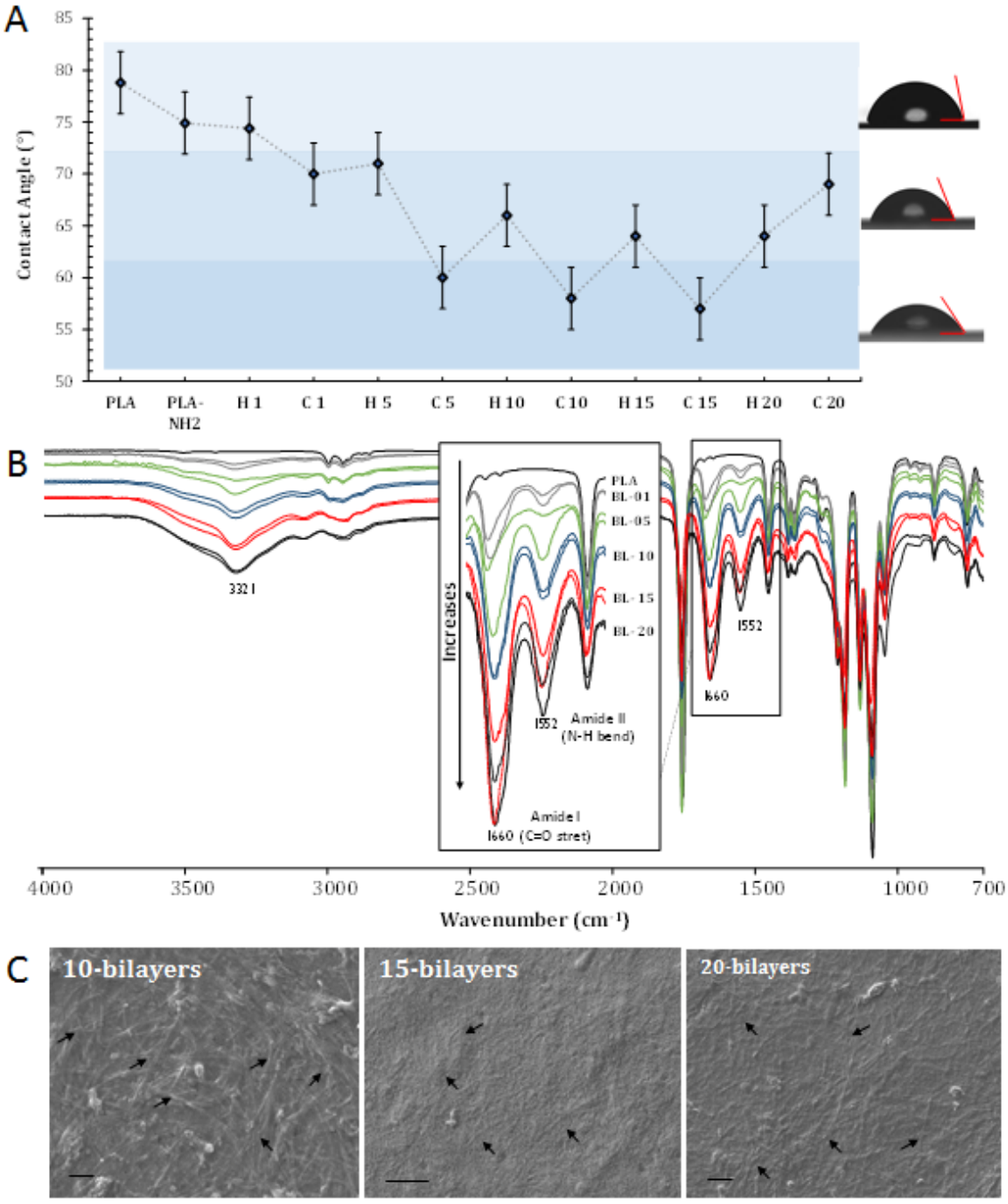

Figure 2. **A.** Water contact angles of PLLA substrates surface after hep/coll layers depositions by LbL technique. **B**. FTIR-ATR spectra of control and layered surfaces with 1 (green), 5 (grey), 10 (blue), 15 (red) and 20 (black) films of hep/coll. Solid line corresponds to heparin and dash-

line to collagen spectra, as the outermost layer. **C.** SEM micrographs of 10, 15 and 20 hep/coll films morphology. Scale bars correspond to 2 μm.

FTIR-ATR spectra of 1-layer of heparin (H1) layer showed two new peaks at 1674 and 1552 cm^{-1} (not detected on the control group) demonstrating the amide bond formation between the amino groups superficially exposed on the aminolysed surface and the carboxyl groups of heparin. Moreover the collagen α-helix presents three typical absorptions: (1) N-H stretching in the vibration frequency range 3290-3300 cm^{-1}, (2) C=O stretching (amide I) between 1648-1660 cm^{-1} and (3) N-H deformation (amide II) at 1540-1580 cm^{-1}. Because the amide bond position is dependent on the secondary structure of the species, as the number of hep/coll films increased, the asymmetric stretching of the carbonyl group (amide I at 1674 cm^{-1}) was slightly shifted to 1658 cm^{-1} by the presence of the strong absorption of collagen amide I at 1633 cm^{-1} [25]. This effect was particularly evident after the deposition of 5 bilayers of hep/coll. The intensity of N-H bending of amino groups (amide II) vibration at 1552 cm^{-1} increased when 5-layers of collagen were deposited (C5). Moreover, the peak of the symmetric $–NH^{3+}$ deformations - also present in this region – increased its intensity correlating with elevated collagen charge density. Two new peaks at 3322 cm^{-1} and 3089 cm^{-1} associated with the N-H stretching were detected on surfaces coated with 5 collagen layers up to 20 multilayers of heparin and collagen. The presence of 20-bilayers of hep/coll showed a decrease of the peaks associated with amide I and II as well as N-H stretching, when compared to the surface coated with 15-bilayers (C15). A new peak at 997 cm^{-1} attributed to the C−O−S stretching vibration was found in the spectrum of the 20-layer of heparin (H20) sample. Therefore, the deposition of multiple hep/coll films (more than 15 bilayers) resulted in the decrease of the characteristic peaks associated with collagen such as amide I and II. It may be possible that the presence of heparin, which is a strong polyanion, might orient and

bind the positively charged collagen deposited layers until the net charge of collagen turns negative, restricting the hep/coll assembly process [26-27]. Since the electrostatic interactions dominate the species absorption, electrostatic repulsion is considered the main contribution between the negative charged heparin and the outermost polyelectrolyte layer. The saturation of the superficial charge density of the hep/coll film may result in the superficial charge reversion and consequently, in the repulsion between the charges of both polyelectrolytes groups. This effect decreases the protein absorption and constrains the LbL assembly process [28]. Therefore the LbL process of heparin and collagen started to be reversed with the assembly of more than 15 layers of hep/coll. Thus, multilayers of heparin and collagen were chemically stable up to 10-bilayers of hep/coll. Moreover, water contact angle results have demonstrated continued hydrophilic properties in surfaces coated with 10 bilayers of hep/coll. The surface topography of collagen and heparin multilayers was observed by SEM analysis (Figure 2C). The presence of collagen molecules that had self-assembled into fibrils was found in the different samples. Specifically, highly fibrous meshes were observed on the surfaces coated with 10 hep/coll bilayers. Smoother surface topography and absence of aggregates were obtained with increasing hep/coll multilayers from 10 up to 20-bilayers. The macromolecular organisation of collagen into fibrillar structures and enhanced surface hydrophility is demonstrated with increasing numbers of hep/coll bilayers. Electrostatic interactions between heparin and atelocollagen have been reported to enhance parallel accretion during co-fibrillogenesis, since collagen microfibrils are stabilised by heparin action as cross-linker molecule inside the co-fibrils [10]. The absence of the telopeptides on atelocollagen molecules (pepsin-digested soluble collagen) may favour the intercalation of highly negatively charged molecules such as heparin at a specific binding sites [10] contributing to the limitations on the number of bilayers that can be assembled.

Results of static contact angle (hydrophilic properties), FTIR-ATR (chemical structure of films) and SEM (morphology of films) analysis demonstrated that the proper surface charge density is found between 10 and 15 bilayers of hep/coll, Ten bilayers of hep/coll were selected as the optimal number of hep/coll multilayers to be assembled onto polymeric substrates for cell studies. Figure 3A shows collagen and heparin concentrations present in 1 and 10-bilayers of hep/coll. Heparin and collagen concentration were determined by Taylor's blue and Sirius red titration method, respectively. The heparin concentration present in just one layer of heparin was 0.07 ± 0.03 µg/cm^2 whilst in 10-layers was 0.91 ± 0.04 µg/cm^2, nine times greater than in one layer of heparin. Moreover, the concentration held in one layer of collagen was 0.15 ± 0.02 µg/cm^2 while in 10 layers was 1.00 ± 0.02 µg/cm^2.

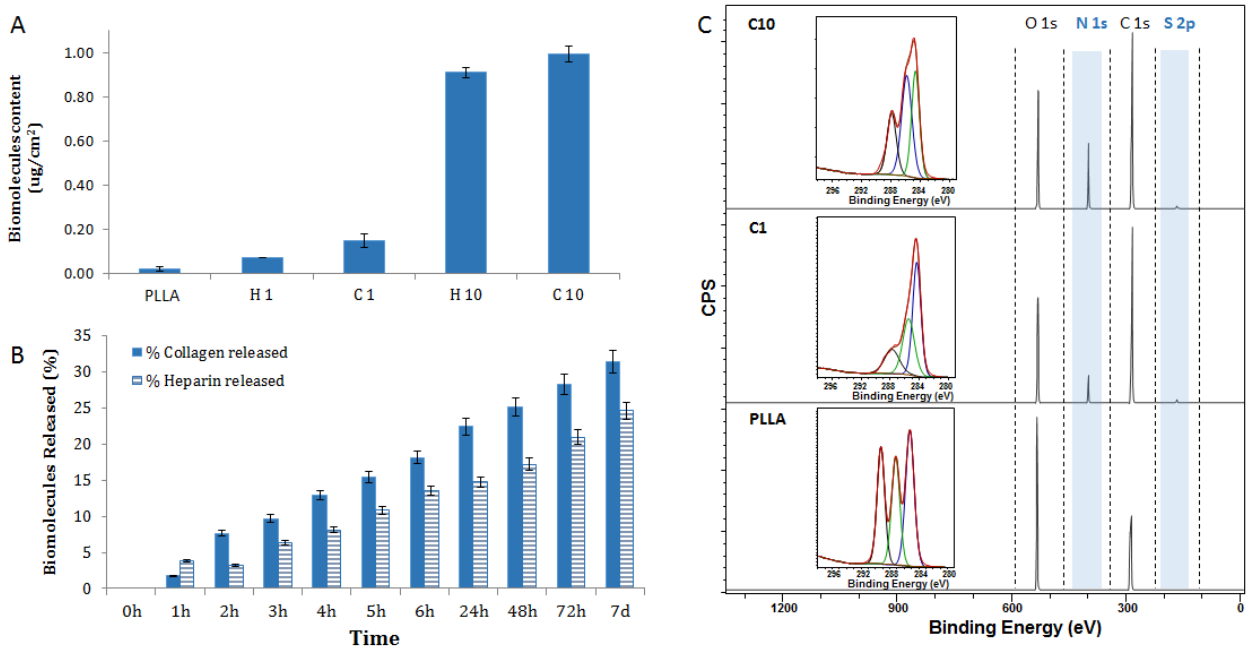

Figure 3. A. Bar diagrams of collagen and heparin concentration of substrates coated with 1 (C1) and 10 films (C10) of hep/coll by LbL technique. **B.** Bar diagrams of the biomolecules released at several time points. Data is presented as mean ± standard deviation of n=5 samples.

C. Survey XPS spectra of uncoated substrate and after coating by LbL assembly at 1 and 10 films of hep/coll.

XPS spectra confirmed the successful deposition of the multilayer coating showing the presence of nitrogen at 400 eV and sulphur at 169 eV that represent the characteristic elements of collagen and heparin (Figure 3C). Furthermore, as shown in Table 1, the content of nitrogen increased significantly from 1 to 10 heparin/collagen bilayers (from 4.3 % ± 2.3 % to 10.7 % ± 0.2 %), evidencing the increase in the thickness of the resulted multilayer. The presence of sulphur was observed after 1 heparin/collagen bilayer, revealing the presence of the heparin coating. No increase was detected as the numbers of bilayers were increased, because the XPS spectra were obtained when the collagen was the top layer (nitrogen is the characteristic element of collagen whilst sulphur is the characteristic element from heparin). Furthermore, the insets, showing the high resolution spectrum of C1s from PLLA samples to C10, revealed the formation of the amide bond at (288.7 eV) from the collagen polyelectrolytes.

Table 1. Atomic concentration of the characteristic element (N, C, O, S) detected on the substrate surface uncoated and coated with 1 and 10 films of hep/coll by LbL technique. Data is presented as mean ± standard deviation of n=3 samples.

	C (At %)	**O (At %)**	**N (At %)**	**S (At %)**
PLLA	65.0 ± 0.4	34.7 ± 0.5	-	-
C1	73.9 ± 1.9	18.1 ± 0.8	4.3 ± 2.3	0.5 ± 0.2
C10	71.3 ± 0.4	17.3 ± 0.4	10.7 ± 0.2	0.7 ± 0.1

Effect of heparin-collagen multilayers on osteogenic cells line (MC3T3-E1). In vitro studies were assessed by MC3T3-E1 cell adhesion on PLLA substrates coated with 1 and 10 bilayers of

hep/coll and incubated for 24, 48 and 72 hours. Cell viability on substrates containing hep/coll films showed no significant differences compared to the control substrates, after 24 hours of incubation. However, a significant decrease in cell number was detected on substrates coated with hep/coll films compared to PLLA alone after 48 and 72 hours (Figure 4A). The presence of small amounts of heparin (H1) did not show significant differences ($p < 0.05$) in terms of cell adhesion compared to untreated polymeric substrates during 72 hours of cell culture. Increased cell adhesion has been related to decreased hydrophobicity [29], finding as optimal contact angle for cell adhesion about 60°. However, after 48 and 72 hours of incubation, lower cell adhesion was found on the more hydrophilic substrate C10 (~62°) in comparison to PLLA control (~80°). Cellular functions are triggered by the surface topography as well as by the chemistry and concentration of immobilised biomolecules on the substrate [30], therefore changes to surface physical-chemical properties (such as stiffness, topography, chemical composition, wettability, etc) due to biomolecule organisation, release and layer dissociation has a direct impact on cell adhesion and morphology. In addition, the number of adherent cells decreases by adding a single collagen layer (C1) and with further increase of hep/coll multilayers (C10) after 48 and 72 hours of incubation. Heparin is capable of interacting with several proteins involved in the bone formation process [31-32], including type I collagen, where the interaction involves a binding site in the telopeptide region [9, 33]. A potential blocking of cell recognition sites of collagen by heparin intercalation [34] might also impact on cellular attachment [35].

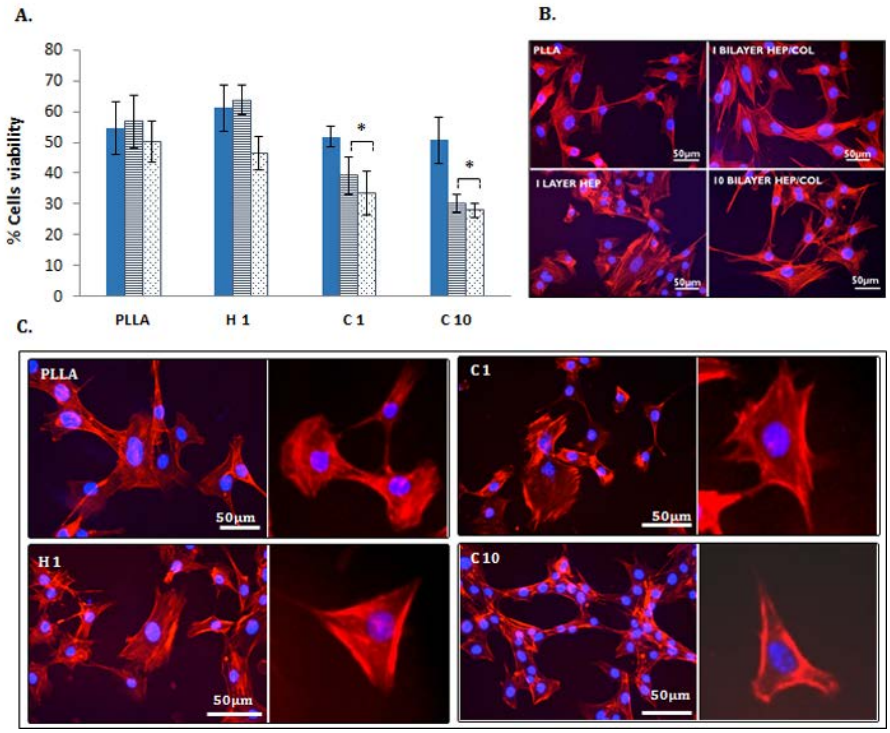

Figure 4. A. MC3T3-E1 cell numbers on PLLA substrates coated with hep/coll films through LbL technique after 24 (blue), 48 (line pattern) or 72 hours (grid pattern). Data is presented as mean ± standard deviation of n=5 samples. (*) corresponds to a significant difference compared to PLLA control surface, for $p<0.05$. **B.** Fluorescence micrographs of cells attached on control substrates (PLLA), prepared heparin layer (H1), prepared hep/coll bilayer (C1) and 10-bilayers of hep/coll (C10) after 48 hours and **C.** 72 hours of incubation. Scale bars correspond to 50 µm.

Cell adhesion provides mechanical linkage to a surface and influences not only cell shape but also proliferation, viability, motility and differentiation. Cell size and shape has also been shown to be inversely correlated with cell proliferation and migration [15, 36-37]. Fluorescence microscopy analysis of cells attached to the different surfaces after 48 hours (Figure 4B) revealed that they were well spread, had extensive interactions and adopted a polygonal morphology on the different surfaces. The cells were also widely distributed over the entire surfaces showing no regional variations. Analysis of cell morphology after 48 and 72 hours on the different surfaces

revealed no significant differences in terms of cell area (Figure 4C), indicating that collagen and heparin multilayers may not directly influence bone cellular phenotype through direct cytoskeletal organisation. The average cells area was measured for the various surfaces obtaining the following results: 1) the control group was 2264 ± 776 μm^2, 2) heparin prepared layer was 1236 ± 492 μm^2, 3) hep/coll prepared bilayer was 1568 ± 852 μm^2, and 4) 10-bilayers of hep/coll was 1228 ± 612 μm^2. The differences of average cell area were not significant between hep/coll films and the control, using the ANOVA one-way test ($p < 0.05$).

MC3T3-E1 activity on the different hep/coll coated surfaces may be influenced by the combination of two different effects: 1) molecular topography resulting from the concentration and organisation of heparin and collagen biomolecules on the polymeric substrate, and 2) the direct effect of localised heparin concentrations on cell function through control of growth factor availability/activity. The chemistry and topography of a surface can trigger specific cellular behaviour including cell spreading, migration, proliferation and differentiation, which eventually may affect the rate and quality of new tissue formation [38-39]. The increase of hep/coll multilayers on the surface promoted a less fibrillary surface at the microscale as observed by SEM micrographs. Since bone cells are sensitive to slight differences in surface roughness and surface chemistry, the modifications in surface texture observed after biomolecule depositions may affect cell response. Cell numbers began to decrease over time on the surfaces containing increased numbers of hep/coll films. This effect may be caused by changes in surface morphology associated to the biomolecules desorption.

Effect of heparin-collagen multilayers on hMSCs in vitro. Studies of cellular activity were carried out using human bone marrow-derived mesenchymal stem cells (hMSCs) to assess the effect of hep/coll multilayers on cellular fate. Substrates coated with hep/coll layers (heparin

alone, hep/coll bilayer and 10-films of hep/coll) and controls were incubated for 1, 3, 7, 14 and 28 days. Figure 5A shows the hMSCs adhesion results on different substrates coated with hep/coll films in the short and long-term. In general, cell numbers did not show significant differences ($p<0.05$) between substrates coated with of hep/coll layers and control, except for surfaces with a heparin layer after 24 hour that showed increased cell number. Similarly, to MC3T3 results , hMSC numbers were reduced by introducing a layer of collagen (C1) and further increases of deposited hep/coll films (C10) in the short-term experiments. The lowest values for cell numbers were observed in surfaces coated with 10-bilayers of hep/coll at 7 days, in comparison to controls (PLLA) and the rest of substrates (H1 and C1). This effect was diminished in the long-term (14 and 28 days), since no significant differences were observed between the hep/coll films and control surfaces.

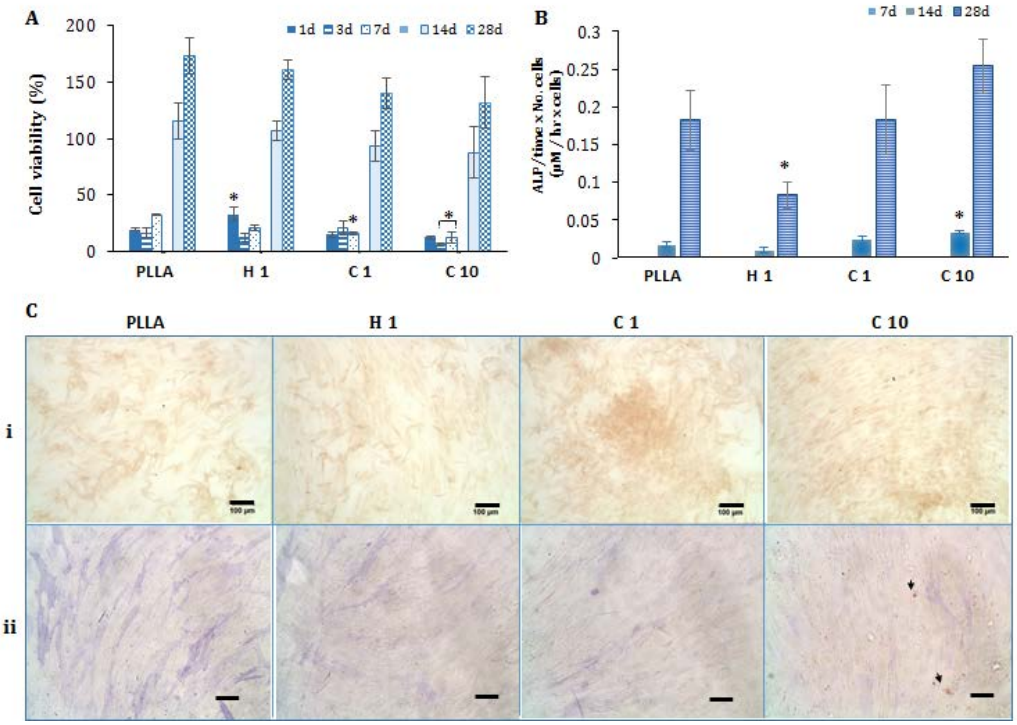

Figure 5. A. Human MSCs viability. Cells were seeded at 20,000 cells/well and cultured for 1, 3 and 7, 14 and 28 days days on control substrates (PLLA), and prepared with a heparin layer

(H1), a hep/coll bilayer (C1) and 10 bilayers of hep/coll (C10). **B.** Alkaline phosphatase activity normalised to cell numbers adhered onto non-coated and hep/coll coated substrates after 7, 14 and 28 days of incubation. Data is presented as mean ± standard deviation of n=3 samples. (*) corresponds to a significant difference compared to PLLA control surface, for $p<0.05$. **C.** ALP staining of differentiating hMSCs and calcium deposition on the different surfaces after i) 7 days and ii) 28 days of incubation. ALP-positive cells are shown in purple; mineral deposits pointed with black arrows were stained orange/red by Alizarin-Red. Scale bars correspond to 100 µm.

Type I collagen, the main protein in the ECM, is known to stimulate the adhesion, proliferation and differentiation of hMSCs [40], whilst heparin influences cell adhesion and induces osteogenic differentiation of hMSC [41-42]. Specific heparin-binding short peptide sequences have been also investigated to improve the osteoblastic mineralisation (FHRRIKA) [31] and adhesion (KRSR) [32]. However, the effect induced by the combination of both biomolecules on hMSCs activity is poorly understood. Soluble collagen is prone to the intercalation of highly negatively charged molecules such as heparin in the specific binding site in the telopeptide region [10]. Studies investigating collagen peptides have shown a greater heparin affinity in the C-terminal binding site than N-terminal binding site, only when the consensus sequence in surrounding residues are included [10, 33] . The specific collagen motif recognized by the α2-I domain is contained within the six-residue sequence GFOGER (O = hydroxyproline) [34]. The blocking of the collagen cell-binding domain by heparin intercalation at a specific binding site in the telopeptides region of collagen triple helix could prevent specific cell binding, decreasing the number of cells attached to the surface.

Alkaline phosphatase activity (ALP) was evaluated to assess potential hMSCs differentiation on hep/coll films after 7, 14 and 28 days of incubation (figure 5B). In general, ALP activity

increased significantly on all substrates after 14 and 28 days. Surfaces containing 1 and 10 bilayers hep/coll (C1 and C10) supported high levels of ALP activity as PLLA control at day 14 and 28; whilst substrates containing a single layer of heparin (H1) showed significantly less ALP expression. Although, the result indicates that heparin has a direct impact on potential hMSC's differentiation, the combination of collagen and heparin and further increases of multiple hep/coll layer may help osteogenic differentiation and stimulate osteoblast formation.

ALP activity was stained purple/blue by Fast Blue RR whilst mineral deposits were coloured orange/red by Alizarin Red. Figure 5C shows the hMSCs cellular differentiation and mineral deposition after 14 days of incubation. After 7 days of incubation, hMSC's showed negative stain of ALP activity and no mineral deposits were observed. However, after 28 days of culture, control PLLA and hep/coll coated substrates expressed ALP, evidenced purple staining. Mineral deposition was detected on samples coated with 10 bilayers of hep/coll (C10). Therefore, PLLA substrates coated with hep/coll multicoatings provide a more osteoinductive and osteoconductive environment for hMSC's.

Results presented in this work have evidenced that the presence of a single heparin layer may stimulate an initial cell adhesion of MC3T3 and hMSC's, and prevent hMSC's differentiation in the mid-long term. However, by combining electrostatically heparin with collagen molecules into multiple layers, for cell differentiation and mineral deposition is promoted.

CONCLUSIONS

Polyelectrolyte complex films made of collagen and heparin were successfully built–up on PLLA films using the LBL self-assembly method. PLLA substrates functionalised with isolated collagen type I and heparin presented a critical charge density close to 10-bilayers of hep/coll.

Heparin has been shown to play an important role on collagen molecules self-assembling into fibrils and in the stabilization of the fibrous collagen layer. Cell culture analysis using MC3T3-E1 osteoblasts revealed that cell viability is reduced by increasing the number of deposited hep/coll films in the short-term. However, this work demonstrates that the presence of electrostatic hep/coll films do not inhibit hMSCs adhesion, self-renewal potential, lineage commitment, differentiation or mineralization but support contrasting and complex effects on short and long-term cells behaviour. The presence of multiple hep/coll bilayers electrostatically deposited on polymeric substrates reduces the initial cell adhesion whilst stimulating cellular differentiation and mineralisation at long term, providing a more osteoinductive and osteoconductive environment for hMSC's.

AUTHOR INFORMATION

Corresponding Author

* Ana M. Ferreira and Mark A. Birch

Email: ana.ferreira-duarte@ncl.ac.uk and mab218@cam.ac.uk

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

1. Agrawal, C. M.; Niederauer, G. G.; Athanasiou, K. A., Fabrication and characterization of PLA-PGA orthopedic implants. *Tissue Eng.* **1995,** *1* (3), 241-252.

2. Sabir, M. I.; Xu, X.; Li, L., A review on biodegradable polymeric materials for bone tissue engineering applications. *J. Mater. Sci.* **2009,** *44* (21), 5713-5724.

3. Ai, H.; Jones, S. A.; Lvov, Y. M., Biomedical applications of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles. *Cell Biochem. Biophys.* **2003,** *39* (1), 23-43.

4. Rother, S.; Salbach-Hirsch, J.; Moeller, S.; Seemann, T.; Schnabelrauch, M.; Hofbauer, L. C.; Hintze, V.; Scharnweber, D., Bioinspired collagen/glycosaminoglycan-based cellular microenvironments for tuning osteoclastogenesis. *ACS Appl. Mater. Interfaces* **2015,** *7* (42), 23787-23797.

5. De Villiers, M. M.; Otto, D. P.; Strydom, S. J.; Lvov, Y. M., Introduction to nanocoatings produced by layer-by-layer (LbL) self-assembly. *Adv. Drug Del. Rev.* **2011,** *63* (9), 701-715.

6. You, J.; Raghunathan, V. K.; Son, K. J.; Patel, D.; Haque, A.; Murphy, C. J.; Revzin, A., Impact of nanotopography, heparin hydrogel microstructures, and encapsulated fibroblasts on phenotype of primary hepatocytes. *ACS Appl. Mater. Interfaces* **2014,** *7* (23), 12299-12308.

7. Crespilho, F. N.; Zucolotto, V.; Oliveira Jr, O. N.; Nart, F. C., Electrochemistry of layer-by-layer films: a review. *Int. J. Electrochem. Sci* **2006,** *1* (5), 194-214.

8. Decher, G.; Hong, J. D. In *Buildup of ultrathin multilayer films by a self-assembly process, 1 consecutive adsorption of anionic and cationic bipolar amphiphiles on charged surfaces*, Makromolekulare Chemie. Macromolecular Symposia, Wiley Online Library: **1991**; pp 321-327.

9. Stamov, D.; Grimmer, M.; Salchert, K.; Pompe, T.; Werner, C., Heparin intercalation into reconstituted collagen I fibrils: Impact on growth kinetics and morphology. *Biomaterials* **2008,** *29* (1), 1-14.

10. Stamov, D. R.; Nguyen, T. A. K.; Evans, H. M.; Pfohl, T.; Werner, C.; Pompe, T., The impact of heparin intercalation at specific binding sites in telopeptide-free collagen type I fibrils. *Biomaterials* **2011,** *32* (30), 7444-7453.

11. Hannink, G.; Geutjes, P. J.; Daamen, W. F.; Buma, P., Evaluation of collagen/heparin coated TCP/HA granules for long-term delivery of BMP-2. *J. Mater. Sci. Mater. Med.* **2013,** *24* (2), 325-332.

12. Lanfer, B.; Seib, F. P.; Freudenberg, U.; Stamov, D.; Bley, T.; Bornhäuser, M.; Werner, C., The growth and differentiation of mesenchymal stem and progenitor cells cultured on aligned collagen matrices. *Biomaterials* **2009,** *30* (30), 5950-5958.

13. Tang, Z.; Wang, Y.; Podsiadlo, P.; Kotov, N. A., Biomedical applications of layer-by-layer assembly: from biomimetics to tissue engineering. *Adv. Mater.* **2006,** *18* (24), 3203-3224.

14. Gentile, P.; Carmagnola, I.; Nardo, T.; Chiono, V., Layer-by-layer assembly for biomedical applications in the last decade. *Nanotechnology* **2015,** *26* (42), 422001.

15. Chen, J.; Chen, C.; Chen, Z.; Chen, J.; Li, Q.; Huang, N., Collagen/heparin coating on titanium surface improves the biocompatibility of titanium applied as a blood-contacting biomaterial. *J. Biomed. Mater. Res., Part A* **2010,** *95* (2), 341-349.

16. Chen, J. L.; Li, Q. L.; Chen, J. Y.; Chen, C.; Huang, N., Improving blood-compatibility of titanium by coating collagen–heparin multilayers. *Appl. Surf. Sci.* **2009,** *255* (15), 6894-6900.

17. Chen, J.; Huang, N.; Li, Q.; Chu, C. H.; Li, J.; Maitz, M. F., The effect of electrostatic heparin/collagen layer-by-layer coating degradation on the biocompatibility. *Appl. Surf. Sci.* **2016,** *362*, 281-289.

18. Chou, C.-C.; Hsin, S.-W.; Lin, H.-C.; Yeh, C.-H.; Wu, R.; Cherng, W.-J., Oxidized dopamine as the interlayer between heparin/collagen polyelectrolyte multilayers and titanium substrate: An investigation of the coating's adhesion and hemocompatibility. *Surf. Coat. Technol.* **2016**.

19. Jin, K.; Li, B.; Lou, L.; Xu, Y.; Ye, X.; Yao, K.; Ye, J.; Gao, C., In vivo vascularization of MSC-loaded porous hydroxyapatite constructs coated with VEGF-functionalized collagen/heparin multilayers. *Sci. Rep.* **2016,** *6*.

20. Lu, S.; Zhang, P.; Sun, X.; Gong, F.; Yang, S.; Shen, L.; Huang, Z.; Wang, C., Synthetic ePTFE grafts coated with an anti-CD133 antibody-functionalized heparin/collagen multilayer with rapid in vivo endothelialization properties. *ACS Appl. Mater. Interfaces* **2013,** *5* (15), 7360-7369.

21. Ferreira, A. M.; Gentile, P.; Sartori, S.; Pagliano, C.; Cabrele, C.; Chiono, V.; Ciardelli, G., Biomimetic soluble collagen purified from bones. *Biotechnol. J.* **2012,** *7* (11), 1386-1394.

22. Gong, Y.; Zhu, Y.; Liu, Y.; Ma, Z.; Gao, C.; Shen, J., Layer-by-layer assembly of chondroitin sulfate and collagen on aminolyzed poly (L-lactic acid) porous scaffolds to enhance their chondrogenesis. *Acta Biomater.* **2007,** *3* (5), 677-685.

23. Zhu, Y.; Chan-Park, M. B., Density quantification of collagen grafted on biodegradable polyester: its application to esophageal smooth muscle cell. *Anal. Biochem.* **2007,** *363* (1), 119-127.

24. Hartwig, A.; Mulder, M.; Smolders, C. A., Surface amination of poly (acrylonitrile). *Adv. Colloid Interface Sci.* **1994,** *52*, 65-78.

25. Pieper, J. S.; Hafmans, T.; Van Wachem, P. B.; Van Luyn, M. J. A.; Brouwer, L. A.; Veerkamp, J. H.; Van Kuppevelt, T. H., Loading of collagen-heparan sulfate matrices with bFGF promotes angiogenesis and tissue generation in rats. *J. Biomed. Mater. Res.* **2002,** *62* (2), 185-194.

26. Hoogeveen, N. G.; Cohen Stuart, M. A.; Fleer, G. J.; Böhmer, M. R., Formation and stability of multilayers of polyelectrolytes. *Langmuir* **1996,** *12* (15), 3675-3681.

27. Muller, M.; Rieser, T.; Dubin, P. L.; Lunkwitz, K., Selective interaction between proteins and the outermost surface of polyelectrolyte multilayers: influence of the polyanion type, pH and salt. *Macromol. Rapid Commun.* **2001,** *22* (6), 390-395.

28. Ladam, G.; Schaaf, P.; Cuisinier, F. J. G.; Decher, G.; Voegel, J.-C., Protein adsorption onto auto-assembled polyelectrolyte films. *Langmuir* **2001,** *17* (3), 878-882.

29. Dowling, D. P.; Miller, I. S.; Ardhaoui, M.; Gallagher, W. M., Effect of surface wettability and topography on the adhesion of osteosarcoma cells on plasma-modified polystyrene. *J. Biomater. Appl.* **2010**.

30. Stevens, M. M.; George, J. H., Exploring and engineering the cell surface interface. *Science* **2005,** *310* (5751), 1135-1138.

31. Rezania, A.; Healy, K. E., Biomimetic Peptide Surfaces That Regulate Adhesion, Spreading, Cytoskeletal Organization, and Mineralization of the Matrix Deposited by Osteoblast-like Cells. *Biotechnol. Progr.* **1999,** *15* (1), 19-32.

32. Dee, K. C.; Andersen, T. T.; Bizios, R., Design and function of novel osteoblast-adhesive peptides for chemical modification of biomaterials. *J. Biomed. Mater. Res.* **1998,** *40* (3), 371-377.

33. Doss-Pepe, E.; Deprez, P.; Silva, T.; Inestrosa, N. C.; Kirkpatrick, A.; Ramshaw, J. A. M.; Brodsky, B., Stepwise construction of triple-helical heparin binding sites using peptide models. *Biochim. Biophys. Acta* **2004,** *1698* (2), 187-195.

34. Knight, C. G.; Morton, L. F.; Peachey, A. R.; Tuckwell, D. S.; Farndale, R. W.; Barnes, M. J., The Collagen-binding A-domains of Integrins α1β1 and α2β1recognize the same specific amino acid sequence, GFOGER, in native (Triple-helical) collagens. *J. Biol. Chem.* **2000,** *275* (1), 35-40.

35. Mhanna, R. F.; Vörös, J.; Zenobi-Wong, M., Layer-by-layer films made from extracellular matrix macromolecules on silicone substrates. *Biomacromolecules* **2011,** *12* (3), 609-616.

36. Madri, J. A.; Pratt, B. M.; Yannariello-Brown, J., Matrix-driven cell size change modulates aortic endothelial cell proliferation and sheet migration. *Am. J. Pathol.* **1988,** *132* (1), 18.

37. Ingber, D. E.; Madri, J. A.; Folkman, J., Endothelial growth factors and extracellular matrix regulate DNA synthesis through modulation of cell and nuclear expansion. *In Vitro Cell. Dev. Biol.* **1987,** *23* (5), 387-394.

38. Boyan, B. D.; Hummert, T. W.; Dean, D. D.; Schwartz, Z., Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials* **1996,** *17* (2), 137-146.

39. Middleton, J. C.; Tipton, A. J., Synthetic biodegradable polymers as orthopedic devices. *Biomaterials* **2000,** *21* (23), 2335-2346.

40. Liu, G.; Hu, Y. Y.; Zhao, J. N.; Wu, S. J.; Xiong, Z.; Lu, R., Effect of type I collagen on the adhesion, proliferation, and osteoblastic gene expression of bone marrow-derived mesenchymal stem cells. *Chin. J. Traumatol.* **2004,** *7* (6), 358-362.

41. Zhu, A.; Zhang, M.; Wu, J.; Shen, J., Covalent immobilization of chitosan/heparin complex with a photosensitive hetero-bifunctional crosslinking reagent on PLA surface. *Biomaterials* **2002,** *23* (23), 4657-4665.

42. Benoit, D. S. W.; Anseth, K. S., Heparin functionalized PEG gels that modulate protein adsorption for hMSC adhesion and differentiation. *Acta Biomater.* **2005,** *1* (4), 461-470.

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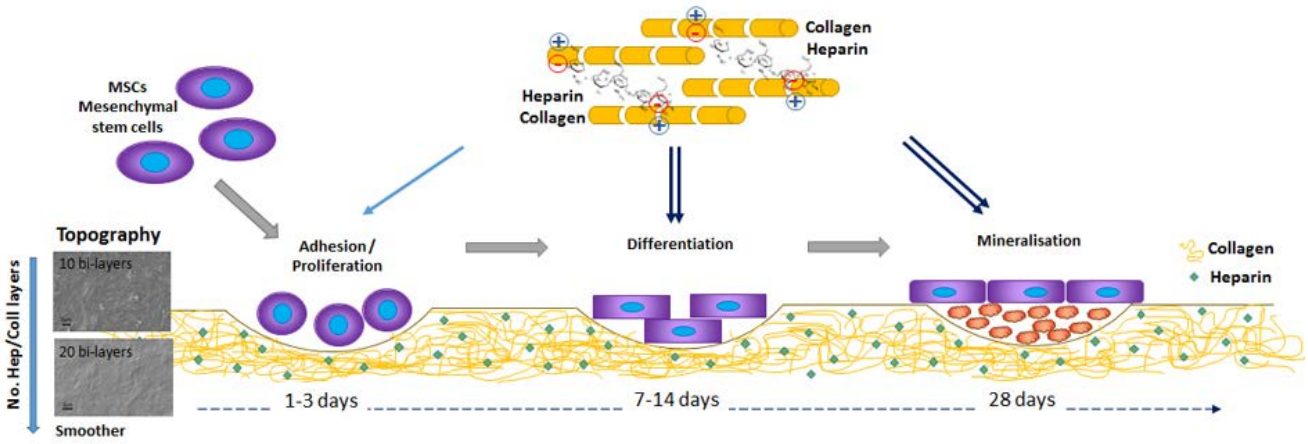
MSCs
Mesenchymal
stem cells
Collagen
Heparin
Heparin
Collagen
Topography
10 bi-layers
20 bi-layers
No. Hep/Coll layers
Smoother
Adhesion /
Proliferation
Differentiation
Mineralisation
Collagen
Heparin
1-3 days
7-14 days
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