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# Crosslinked layered surfaces of heparin and poly(L-lysine) enhance mesenchymal stromal cells behavior in the presence of soluble interferon gamma

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## Abstract

Human mesenchymal stromal cells (hMSCs) are multipotent cells that have been proposed for the treatment of immune-mediated diseases. Culturing hMSCs on tissue culture plastic reduces their therapeutic potential in part due to the lack of extracellular matrix components. The aim of this study is to evaluate multilayers of heparin and poly(L-lysine) (HEP/PLL) as a bioactive surface for hMSCs stimulated with soluble interferon gamma (IFN-γ). Multilayers were formed, via layer-by-layer assembly, with HEP as the final layer and supplemented with IFN-γ in the culture medium. Multilayer construction and chemistry were confirmed using Azure A staining, quartz crystal microbalance (QCM), and X-ray photoelectron spectroscopy. hMSCs adhesion, viability, and differentiation, were assessed. Results showed that (HEP/PLL) multilayer coatings were poorly adhesive for hMSCs. However, performing chemical crosslinking using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC/NHS) significantly enhanced hMSCs adhesion and viability. The immunosuppressive properties of hMSCs cultured on crosslinked (HEP/PLL) multilayers were confirmed by measuring the level of indoleamine 2,3-dioxygenase (IDO) secretion. Lastly, hMSCs cultured on crosslinked (HEP/PLL) multilayers in the presence of soluble IFN- γ successfully differentiated towards the osteogenic and

adipogenic lineages as confirmed by Alizarin red, and oil-red O staining, as well as alkaline phosphatase activity. This study suggests that crosslinked (HEP/PLL) films can modulate hMSCs response to soluble factors, which may improve hMSCs-based therapies aimed at treating several immune diseases.

KEYWORDS: Layer-by-layer, Human mesenchymal stromal cells, poly(L-lysine), Heparin, Interferon gamma.

## Introduction

Human mesenchymal stromal cells (hMSCs) are of special interest for cellular therapy programs (Ramos et al. 2016). This type of cells are pluripotent which are able to differentiate into mesodermal lineage cells, including adipocytes, osteoblasts, and chondrocytes [2][3]. HMSCs therapeutic behavior is affected by the surrounding microenvironment, including growth factors, the extracellular matrix (ECM) and contact with other cells (Watt and Huck 2013)(Hynes 2009). The ECM is a highly complex nanostructure that play vital roles in the determination, differentiation, proliferation, and survival of cells (Hynes 2009). The ECM structure is filled with the matrix containing glycosaminoglycans (Lortat-Jacob 2009) and proteins (Brizzi, Tarone, and Defilippi 2012), which are known as proteoglycans (Hynes 2009). The proteoglycans in the ECM contribute to the mechanical properties of the matrix and modulate cell behavior (Watt and Huck 2013)(Hynes 2009). There is a need to learn from cell biology, such as what controls cellular differentiation and growth and how ECM components affect cell function (Brizzi, Tarone, and Defilippi 2012).

The layer-by-layer (LbL) deposition of polyelectrolytes has been known as a simple method to generate biologically relevant surfaces by creating nanoscale thin films. The LbL method provides compositional uniqueness of natural or synthetic polymers, such as stimulating a specific signal to cells and enhancing cellular behavior (Gribova, Auzely-Velty, and Picart 2012). LbL involves the alternative absorption of polycations and polyanions to produce films with specific and controlled physical-chemical characteristics by adapting the experimental parameters, such as pH, ionic strength, and polyelectrolyte concentration (Catherine Picart et al. 2005)(Catherine Picart 2008)(Gentile et al. 2015). Several studies have recently investigated cell interactions with multilayers. One study by Growth et al. indicated that (hyaluronic acid (HA)/ (poly-L-lysine (PLL) multilayers composed of 24 layers are able to control stem cell response after chemical cross-

linking (Niepel et al. 2018). This response is in part correlated to the increase in stiffness brought upon by the crosslinking process.

HMSCs interaction with heparin (HEP/PLL) polymeric multilayer composition in the presence or absence of soluble interferon gamma (IFN- γ) has not been studied yet. It has been shown that the immunosuppressive properties of hMSCs relies on the existence of IFN-γ in the microenvironment (Klinker et al. 2017). IFN-γ is a potent pro-inflammatory cytokine that is produced by CD4+ lymphocytes, natural killer cells (NKT) cells, and macrophages. IFN-γ plays essential and complex roles in innate and adaptive immune responses against viral infections, bacteria, protozoa, and graft-versus-host disease (GVHD) (Ijzermans and Marquet 1989)(Liu et al. 2011). A study showed that IFN-γ has the ability to modulate the immune properties and differentiation potential of hMSCs which has a significant anti-proliferative effect (Croitoru-Lamoury et al. 2011). Therefore, there is a need to reduce the anti-proliferative effect of IFN-γ on hMSCs.

Heparin is a highly sulfated glycosaminoglycan that contains negatively charged carboxylate or sulfate groups present in the ECM and surface of cells (Douglas et al. 1997)(Sarrazin et al. 2005). Due to the electrostatic interactions and binding with amino acids, heparin plays a role in cellular functions such as cell adhesion, proliferation, differentiation, migration, and inflammation (Paluck, Nguyen, and Maynard 2016)(Dinoro et al. 2019). Furthermore, heparin is well known for its anticoagulant properties, but apart from this ability, heparin has the ability to bind ECM proteins, such as collagen and thus plays an important role in organizing the structure and composition of the ECM. Many studies showed that heparin can prevent proteolytic cleavage of IFN- γ and can improve IFN- γ signaling (Douglas et al. 1997)(Lortat-Jacob, Baltzer, and Grimaud 1996). In addition, PLL is a biocompatible polycation with a large amount of active amino groups. PLL can adopt different secondary structures (e.g., random coil, b-sheet, or a-helix) depending on the pH of the solution. PLL has been used for many different purposes, such as the study of DNA-Protein interactions, drug delivery, and coating materials to improve cell attachment to plastic and glass surfaces (Shukla et al. 2012). PLL enhances cell's attachment due to electrostatic interaction between negatively-charged ions of the cell membrane and positively-charged surface ions of attachment factors on the culture surface (Pachmann and Leibold 1976)(KRUIJFF and CULLIS 1980).

This research evaluated (HEP/PLL) multilayer substrates as surfaces for hMSC culture. We evaluated the construction and chemistry of the (HEP/PLL) multilayers, as well as their capacity to support hMSCs. Finally, we investigated hMSC viability, adhesion, proliferation, immunosuppressive properties, and differentiation when cultured on (HEP/PLL) multilayers in the presence of soluble IFN-γ.

## Materials and Methods

### Materials

Heparin sodium (HEP) was purchased from Celsus Laboratories, Inc. (Cat. #PH3005). Poly-L-lysine hydrobromide from bovine (Cat. # P2636), poly(ethylenimine) (PEI) (50% solution in Water, Mw ≈ 750 000) (Cat. #P3143), HEPES (Cat. # H3375), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Cat. #106627547), and N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) (Cat. #56485) were purchased from Sigma-Aldrich. Ultrapure water at 18 MΩ·cm was obtained from a Millipore-Sigma™ Direct-Q™ 3 (Cat. #ZRQSVP3US). Tissue culture-treated plates were purchased from Corning Costar (Cat. #07-200- 740). IFN-γ recombinant human protein was purchased from ThermoFisher (Cat. #PHC4031). Human bone-marrow derived mesenchymal stromal cells from two donors purchased from RoosterBio (Cat. #MSC-003), were used between passages 4−6. Donor#1 is a healthy 25-year-old male (Lot. 00174), and donor#2 is a healthy 22-year-old male (Lot. 00178). MEM Alpha (1X) (Cat. #12561-056) and fetal bovine serum (Cat. #12662029) were obtained from Gibco. Penicillin-streptomycin (Cat. #30002CI), and L-glutamine were purchased from Corning (Cat. #25005CI). Azure A was purchased from Thermo Scientific™ (Cat. #AAJ6134614). PrestoBlue™ cell viability assay was purchased from Invitrogen (Cat. #A13261). Hoechst 33 342 was purchased from Invitrogen (Ref. #H3570). ActinRed 555 Ready Probest was purchased from Invitrogen (Ref. #37112). DMEM (Dulbecco's Modified Eagle Medium) high glucose (Cat. #11965092), and DMEM (Dulbecco's Modified Eagle Medium) low glucose were purchased from ThermoFisher (Cat. #11885084). Ascorbic acid (Cat. #50-81-7), β-glycerophosphate from Sigma (Cat. #154804-51-0), Alizarin Red S (Cat. # 130-22-3), dexamethasone (Cat. #50-02-2), insulin (Cat. # I2643), 3-isobutyl-1-methylxanthine (IBMX) (Cat. #28822-58-4, I5879), indomethacin (Cat. #53-86-1) and Oil Red-O (Cat. #O0625) were purchased from Sigma-Aldrich. Alkaline Phosphatase Colorimetric Assay Kit was purchased

from Abcam (ab83369). Micro BCA™ Protein Assay Kit was purchased from Thermofisher (Cat. # 23235).

**(HEP/PLL) multilayers fabrication**

(HEP/PLL) multilayers were constructed by the layer-by-layer technique. PEI (1 mg/mL), HEP (1 mg/mL), and PLL (0.5 mg/mL) were dissolved in a filtered HEPES-NaCl buffer solution (20 mM HEPES pH 7.4, 0.15 M NaCl), and ultrapure water at 18 MΩ·cm was used to prepare the polymeric and wash solutions. Sequential polymeric layers and rinsing were done using manual pipetting on sterile tissue culture-treated plates. Briefly, the process consisted of creating a positive initial layer by depositing PEI solution for 15 minutes to each well of a sterile tissue culture-treated plate and followed by a 3 minutes washing step with HEPES-NaCl buffer solution. HEP was added for 5 minutes; then the HEP solution was removed, collected, and rinsed with HEPES-NaCl buffer solution for 3 minutes. Then PLL was added and subsequently rinsed following the same process. This process was followed until obtaining a total of 13 polymeric layers of (HEP/PLL) (layers ending with HEP). Multilayers were crosslinked with EDC at 25 mg/mL and NHS at 11 mg/mL dissolved in NaCl (0.15 M, pH 5.5 in deionized water) and mixed immediately before use, similar to the process described by Almodovar et al. (Almodóvar et al. 2014). The multilayers were incubated overnight in a humidified incubator at 37 °C. Then the EDC-NHS solution was removed, followed by extensive rinsing with cold 15 M NaCl buffer solution to hydrolyze unreacted cross-linkers. A final wash was done using Dulbecco's phosphate-buffered saline (DPBS)1X without $Ca^{2+}$ and $Mg^{2+}$ for 3 minutes. Substrates were sterilized using ultraviolet light (UV) for 10 minutes to reduce contamination before seeding the cells.

**Experimental design**

In this work, the effects on the cellular response of hMSCs of crosslinked multilayers and the presence or absence of IFN-γ in the culture medium were studied. Three surfaces were assessed, these consisted of a control surface of tissue culture plastic labeled as TCP, a bioactive surface of 13 non-crosslinked (HEP/PLL) multilayers, and 13 crosslinked (CL) of (HEP/PLL) multilayers. These multilayers arrangements will be noted as (HEP/ PLL) and (HEP/ PLL) + CL, respectively. IFN-γ supplemented in cell medium was evaluated at a concentration of 50 ng/mL, and conditions with and without IFN-γ were designated as +IFN-γ and −IFN-γ, respectively. A

50 ng/mL concentration for soluble IFN-γ was selected based on our previous study (Castilla-Casadiego et al. 2019). Time points and the initial number of cells were selected according to the nature of the specific method used.

**Qualitative colorimetric determination of heparin deposited within (HEP/PLL)**

The multilayers were deposited on 24-well plates from Corning Costar (Cat. #07-200- 740). After multilayers deposition, the plate was dried for 1 day in a laminar flow hood. Once the samples were completely dry, 1 mL of Azure A Blue dye in water (80 µg of Azure A in 1 mL water) was added to each well. The absorbance at 620 nm was read using a BioTek Multi-Mode Microplate Reader (Model Synergyt™ 2).

**In-situ deposition of (HEP/PLL) multilayers**

Deposition of polycations, polyanions, and crosslinking was measured by quartz crystal microbalance (QCM-D) with dissipation from Biolin Scientific, Sweden. The multilayer buildup process was described in our previous work (Castilla-Casadiego, Timsina, et al. 2020). Briefly, QCM-D measurements were performed on quartz crystal microbalance. The quartz crystal was cleaned following the manufacturer's protocol. The quartz crystal was immersed in a solution containing 10:2:2 (volume parts) of water, 25% ammonia, and 30% hydrogen peroxide at 75 °C. The clean quartz crystal was settled in the QCM-D chamber, and the flow rate was set up at 100 mL/min. Then the PEI solution was injected continuously for 15 minutes. Then, the HEPES-NaCl buffer was pumped for 3 minutes at the same speed. The HEP solution was injected at the same rate for 5 minutes, followed by the same HEPES-NaCl buffer injection. After that, the PLL solution was injected for 5 minutes at the same rate, followed by the same HEPES-NaCl buffer injection. HEP and PLL were then alternately injected into the chamber (followed by the same HEPES-NaCl buffer injection after each injection) for 13 multilayers. Then, the crosslinking solution was injected into the chamber for 1 h. The inverse frequency shift ($-\Delta f$) and dissipation ($\Delta D$) vs. time curves were recorded.

**X-ray Photoelectron Spectroscopy (XPS).**

X-ray Photoelectron Spectroscopy (XPS) (Versaprobe XPS from Physical electronics) was performed at a photoelectron takeoff angle of 458 on a dry glass substrate, and binding energy scales were referenced to the C1s peak (284.7eV).

**Cell culture**

Human bone-marrow derived mesenchymal stromal cells from two donors were used between passages 4−6. The product specification sheet provided by the vendor shows that these cells demonstrated the ability to undergo adipogenic and osteogenic differentiation and expressed the accepted panel of surface markers (CD45-, CD34-, CD166+, CD90+). hMSCs were grown in MEM Alpha (1X) medium (supplemented with L-glutamine, ribonucleosides, and deoxyribonucleosides) containing 20% fetal bovine serum, 1.2% penicillin-streptomycin, and 1.2% L-glutamine.

**Cell viability on (HEP/PLL) multilayers**

The PrestoBlue™ cell viability assay reagent was used to measure hMSCs viability after 3 days. HMSCs (10000 cells/$cm^2$) were seeded on each condition with and without IFN-γ (TCP, (HEP/PLL), and (HEP/PLL)+CL) on a 96 well-plate, and cell viability was measured as described in our previous works (Castilla-Casadiego, Reyes-Ramos, et al. 2020)(Cifuentes et al. 2020)(Pinzon-Herrera et al. 2020). Briefly, the cell culture medium was removed after 3 days, and 100 µL per well of fresh culture medium containing 10% PrestoBlue reagent. The plate was kept in a humidified incubator with 5% $CO_2$ and 37°C for 3 hours (protected from light). The fluorescence intensity measurement was determined using a BioTek Multi-Mode Microplate Reader (Model Synergy™ 2) with excitation/emission of 560/590 nm. Data were summarized per culture conditions.

Fluorescent staining was performed for the detection of the blue fluorescent dye Hoechst 33 342. This dye stains the nucleic acid because it is permeable to the cell. The red-orange fluorescent dye ActinRedt 555 was detected, which is selective to Actin F (a fundamental component of the cellular cytoskeleton). After three days of culture, the cell medium was removed, and the cells

were fixed with 4% formaldehyde solution for 15 minutes. The samples were washed several times with PBS following by adding Triton X100 for 10 minutes, then washed with PBS 3 times. ActinRedt 555 was first added and incubated for 30 minutes. Then, Hoechst 33 342 was added for 10 minutes and protected from light by aluminum foil. Both dyes washed 5 times with PBS before and after being added. For cell imaging, a Leica inverted fluorescence microscope was used with a standard DAPI filter (excitation/emission of 350/461 nm) for Hoechst 33 342, and a standard TRITC filter (excitation/emission of 540/565 nm) for ActinRedt 55.

**Real-time monitoring of hMSCs behavior on (HEP/PLL) multilayers**

An xCELLigence Real-Time Cell Analyzer (RTCA S16) instrument from ACEA Biosciences Inc. (Cat. #00380601430) was used to measure real-time cell behavior. (HEP/PLL) multilayers were constructed on the wells of an ACEA$^{TM}$ E-Plate L16 (Cat. #00300600890, cell growth area of 0.32 $cm^2$ per well), and hMSCs at a concentration of 5000 cells/$cm^2$ were seeded on each condition (uncovered sensors, non-crosslinked multilayers, and crosslinked multilayers with and without IFN-γ supplemented in the culture medium). The xCELLigence instrument was configured as described in our previous works (Pinzon-Herrera et al. 2020)(Castilla-Casadiego, Timsina, et al. 2020). Briefly, the xCELLigence RTCA S16 was placed inside the incubator to allow the device to warm up for at least 2 hours before use. This step is to avoid any condensation on the station after starting the measurement stage. The RTCA S16 was set up to perform readings every 10 minutes for a period of 72 hours of cell culture.

**Immunomodulatory factor expression of hMSCs on (HEP/PLL) multilayers**

The hMSCs immunomodulatory factor expression was investigated by indoleamine 2, 3-dioxygenase (IDO) activity. In this regard, hMSCs (5000 cells/$cm^2$) with and without IFN-γ supplemented in culture medium were seeded on each condition prepared on a 24 well-plate. The IDO activity was measured after 6 days of culture (changing the cells medium each 2 days) as described in our previous works (Castilla-Casadiego, Reyes-Ramos, et al. 2020)(Cifuentes et al. 2020)(Castilla-Casadiego, Timsina, et al. 2020). Briefly, 100 µL of cell supernatant was mixed with 100 µL standard assay mixture consists of (potassium phosphate buffer (50mM, pH 6.5), ascorbic acid (40 mM, neutralized with NaOH), catalase (200 µg/mL), methylene blue (20 µ M), and L-tryptophan (400 µM)). The mixture was kept at 37°C in a humidified incubator with 5% $CO_2$ for 30 minutes (in a dark environment to protect solutions from light) to allow IDO to

convert L-tryptophan to N-formyl-kynurenine. After that, the reaction was stopped by adding 100 µL trichloroacetic acid 30% (w/vol) and incubated for 30 minutes at 58 °C. Then, 100 µL of mixed cell supernatant/standard transfer into a well of a 96-well microplate, following by adding 100 µL per well of 2% (w/v) p dimethylaminobenzaldehyde in acetic acid. Absorbance was read at 490 nm at the endpoint using a BioTek Synergy™ 2 spectrophotometer (Synergy LX Multi-Mode Reader from BioTek® Model SLXFA).

**Cells Differentiation Assay**

hMSCs differentiation was induced by their culture with differentiation media (Osteogenic and Adipogenic media). Control cultures were grown in regular cell expansion medium. Briefly, hMSCs (10000 cells/$cm^2$) were seeded on each condition prepared on 24 well-plates and grown for 6 days in expansion medium (MEM Alpha (1X) supplemented with L-glutamine, ribonucleosides, and deoxyribonucleosides) containing 20% fetal bovine serum, 1.2% penicillin-streptomycin, and 1.2% L-glutamine) at 37 °C in a humidified incubator with 5% $CO_2$. After the cells reached at least 50% confluency, they were exposed to differentiation medium. For osteogenic differentiation, hMSCs were cultured in the differentiation medium (DMEM low glucose, 10% fetal bovine serum,1% penicillin, 1% L-Glutamin, 50 µM ascorbic acid (50mg/10ml), 10 mM β-glycerophosphate, and 100nM dexamethasone). The medium was replaced every 2-3 days. After 8 days of culture, cells were fixed with 10% formaldehyde. For osteogenic differentiation, Alizarin Red S staining solution was prepared by adding 2g Alizarin Red S in 100 mL water, mixed, and the pH was adjusted to 4.1– 4.3 by the addition of Ammonium Hydroxide, as necessary. Alizarin Red S solution was added to the fixed cells, then incubated at room temperature in the dark (cover with aluminum foil) for 15 minutes. The staining solution was removed and rinsed 3 times with PBS. The samples were analyzed immediately under the microscope to detect calcium deposits. For adipogenic differentiation, hMSCs were cultured in the differentiation medium consisting of DMEM high glucose supplemented with 10% fetal bovine serum, 1% penicillin, 1% L-glutamin, 1 µM dexamethasone, 0.01 mg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 100µM indomethacin. The medium was replaced every 2-3 days. After 8 days of culture, cells were fixed with 10% formaldehyde, stained with 0.5% (w/v) Oil Red O in 100% isopropanol, and incubated

at room temperature for 30 minutes and protected from light. The cell monolayer was washed 2 times with PBS. The sample was analyzed under a light microscope to detect lipid vesicles that appeared in bright red color.

**Alkaline phosphatase (ALP) Assay**

To confirm osteogenic differentiation and to determine the level of activity of the differentiated hMSCs, two assays were performed: alkaline phosphatase (ALP) activity and total protein content (micro-BCA assay). Alkaline phosphatase activity was assessed using the Alkaline Phosphatase Colorimetric Assay Kit. According to standard protocols, after the exposure of cells to osteogenic differentiation medium for 3 days, the samples were washed twice with PBS. Then, 50 µL of the cell lysate with assay buffer was added to a 96 well- plate and 50 µL p-nitrophenyl phosphate (pNPP). The samples incubate at 25°C for 60 minutes, protected from light. In the last step, 20 µL stop solution was added to the wells, then; the plate was read at 405 nm in a microplate reader (Synergy LX Multi-Mode Reader from BioTek® (Model SLXFA). ALP activity was normalized by total protein content (micro-BCA assay). The total protein content was determined according to the protocol of the manufacture 150 µL of the sample was placed in a 96 well-plate with 150 µL of working reagent made from a micro-BCA protein assay kit. The well plate was covered with foil and incubated at 37 °C for 2 hours. Absorbance was read at 562 nm using a BioTek Multi-Mode Microplate Reader (Model Synergy™ 2).

**Statistical Analysis**

The results were presented as mean ± standard error of mean. Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA). A p- value $< 0.05$ was considered statistically significant. The statistical analysis was done using SigmaPlot 14 software.

**Results and Discussion**

**Surface characterization**

The presence of heparin qualitatively determined by Azure A dye method based on study done by Klein et al. (Michael D. Klein, Robert A. Drongowski, Robert J. Linhardt 1982). Figure 1 shows the absorbance values obtained for TCP, (HEP/PLL) multilayers, and (HEP/PLL) + CL

multilayers samples. The color changed from blue to dark purple due to existence of heparin in the bottom of the TCP and (HEP/PLL) + CL . These changes indicate that amount of heparin on (HEP/PLL) + CL is higher than (HEP/PLL) and TCP (p-value < 0.05). These findings comply with a study done by Richert et al. which showed that PLL and hyaluronic acid (PLL/HA) multilayers have an exponential growth that occurred by PLL diffusion into the layers. However, Richert et al. showed that in crosslinked (PLL/HA) multilayers, the diffusion of the PLL was vanished (Richert et al. 2004). The absorbance shows that (HEP/PLL) + CL have two times higher absorbance than (HEP/PLL) which confirms the changing of colors from light purple to dark purple. This result comply with our previous study done by Pinzon-Herrera et al. which showed the increase in absorbance from 1 to 6 bilayers of heparin/collagen multilayers as measured using Taylor's Blue dye (Pinzon-Herrera et al. 2020).

The formation of the (HEP/PLL) multilayers was monitored by QCM-D. QCM-D detects the resonant frequency shift (Δf) and measures the dissipation factor (ΔD) (Marx 2003). QCM-D was used here to investigate physical structures such as adsorbed mass and viscoelastic properties of multilayers (Marx 2003) (Barrantes et al. 2012). Figure 2 shows the normalized frequency shift ($-\Delta f_n/n$) and dissipation (ΔD/n) for the 3rd, 4th, and 7th overtones for the (HEP/PLL) and (HEP/PLL) + CL multilayers. The first 15 minutes correspond to a PEI absorption, followed by a 3 minutes rinsing step are shown in Figure 2. The increase in -Δf and ΔD of every (HEP/PLL) sequential deposition shows that the multilayers slowly deposit onto the quartz crystal (Lin et al. 2011). According to Boulmedais et al. this increase can be considered an exponential increase of thickness for the multilayers (Boulmedais et al. 2006). This indicates that at least one of the two components of the multilayers is diffusing within the multilayers as proposed by Picart et al (C. Picart et al. 2002). It is demonstrated that by increasing of -Δf the mass of deposited multilayers enhanced, whereas the increase of ΔD enhances the viscoelastic structure of the deposited multilayers (Easton et al. 2014). Therefore, adding a rough layer on quartz crystal has a lower -Δf, whereas a dense layer has a higher ΔD value. When HEP is deposited, -Δf and ΔD have a sharp rise with great dispersion between different overtones in both (HEP/PLL) + CL and (HEP/PLL). This indicates that the HEP is a loose and swollen layer. In contrast, the PLL deposited shows not only a slight increase in -Δf but also slight decrease in ΔD. This may occur because of the PLL diffused within the multilayers. In addition, the frequency shifts do not overlap for the different overtones not only in the rinse steps but also during the

adsorption steps. Consequently, this indicates that the Sauerbrey relation is not valid for determining the film mass during rinse and adsorption steps, which indicates that the multilayers are more viscoelastic. Besides, the ratio of the change during the rinse and the adsorption steps in the dissipation factor to the change in frequency ($\Delta D/(-\Delta f/n)$) remains higher than $4 \times 10^{-7}$ $Hz^{-}1$; therefore, the film can be considered soft based on study done by Reviakine et al (Reviakine, Johannsmann, and Richter 2011). After adsorption of the crosslinking solution, the frequency shifts no longer overlap. This indicates that adsorbed films are viscoelastic and that the mass does not follow the Sauerbrey relationship anymore, so a more complex model might be used to determine the adsorbed mass from the frequency shift and dissipation data (Boddohi et al. 2010). Figure 2 (A&C) show crosslinking solution absorbed on multilayers by increasing of the frequency shift and dissipation shift. Based on study done by Niepel et al. the increase of the frequency shift and dissipation shift by adding the crosslinking solutions, increase the roughness of the multilayers (Niepel et al. 2018).

The elemental composition obtained by XPS of (HEP/PLL) and (HEP/PLL) + CL are represented in Figure 3 (A & B). High resolution XPS spectra of C1s (283.4 eV), N1s (398.4 eV), O1s (529.8 eV), and S2P (168.3 eV) are shown in Figure 3 (B). Na and S were mainly the characteristic elements of HEP polysaccharide structure possessing carbonyls ($COO^{-}$), sulphate(-$SO4^{-}$), and hydroxyl group (-OH), while PLL contains a large number of various amino ($NH_2$) group and carboxyl group (-COOH) (Zhang et al. 2017). The presence of more sulfur was detected on the multilayers revealed the presence of HEP (Ferreira et al. 2016). The increase of sulfur peak due to increasing the number of layers shows a successful deposition of HEP. This finding complies with a previous study by Almodovar et al. (Almodóvar et al. 2010). The high-resolution C1s and N1s spectrum indicates the presence of several different chemical species such as amide (288.3 eV and 400.6 eV) (Graf et al. 2009). The crosslinking solution reacted with carboxylate groups of HEP and the amino groups of PLL and results in the formation of amide bonds (Semenov et al. 2009). Moreover, O1s, S2p, C1s, and N1s intensity content decrease in (HEP/PLL) + CL compared to (HEP/PLL), indicating that the presence of the crosslinked multilayers by interacting the amine groups of PLL with the sulfate groups of HEP (Crouzier and

Picart 2009). These findings confirm the results from colorimetric determination of heparin deposited within (HEP/PLL) multilayers.

***PrestoBlue Viability Assay***

The PrestoBlue reagent was used for measuring cell viability after 3 days of culturing hMSCs cells on TCP, (HEP/PLL), and (HEP/PLL) + CL with and without IFN-γ supplemented in the cell culture medium. In the absence of the IFN-γ in culture medium, TCP were selected as the positive control, and its fluorescence intensity was normalized to 100%. All other conditions were assessed in relation to the positive control. Figure 4 (A) shows (HEP/PLL) +CL without IFN-γ has the same viability of the cells compared to TCP, and (HEP/PLL) + CL with IFN-γ has a higher viability about 125% compared to TCP. However, (HEP/PLL) with and without IFN-γ have about 48% (less than half) viability compared to TCP. These findings show that the (HEP/PLL) decrease cell viability. A study done by V. Semenov et al. showed an increase in cell adhesion and growth when using high concentration of crosslinker on (PLL/HA) multilayers (Semenov et al. 2009) which may related to an increase in stiffness of the multilayers. However, (HEP/PLL) + CL have a better ability to increase the cell viability. Figure 4 (A) shows significant differences in cell viability on (HEP/PLL) + CL with IFN-γ compared to without IFN-γ ($p$-value $< 0.05$) which indicates presence of the IFN-γ supplemented in the culture medium has a considerable impact on the conditions with (HEP/PLL) + CL; suggesting that there is a synergistic action of both components. These findings confirm our previous study done by Cifuentes et al. in which they used collagen instead of PLL as polycation polymer in heparin/collagen multilayers (Cifuentes et al. 2020).

We performed a similar study using hMSCs from another donor (donor 2). Regarding the cell viability of donor 2, TCP with IFN-γ has a decrease about 20% compared to the TCP without the IFN-γ ($p$-value$<0.001$) (Supplementary Information Figure S1(A)). Also, Figure S1(A) indicates that there are approximately 25% decrease of the cell viability on the surfaces (HEP/PLL) + CL with and without the IFN-γ compared to the TCP. However, donor 1 shows a higher viability when cells supplemented with IFN-γ which can attribute to the different behavior of donors. Also, the cell viability on the (HEP/PLL) for donor 2 decrease below 50%.

Fluorescence microscopy images of hMSCs nuclei labeled with Hoechst of cells attached to the different surfaces after 72 hours validate the findings about cell viability for both donors (Figure 4 (D) and Supplementary Information Figure S1(B)). It is clear that there is less cell attachment on (HEP/PLL) for both donors.

**Real-time monitoring of cell behavior and proliferation**

In this study, we cultured hMSCs at 25000 cells/cm$^2$ on TCP, (HEP/PLL), and (HEP/PLL) + CL with and without IFN-γ supplemented in the cell culture medium to evaluate the real-time behavior of the cells during the first 72 hours of culture. The action of presence of the IFN-γ in the cell medium was also evaluated. As a control surface, we evaluated growth on uncoated (TCP) biosensors. An xCELLigence RTCA S16 biosensor system was used, which allows the measurement of cell proliferation and growth. This system constantly measures the impedance difference caused by cells attached to microsensors present in culture plates (E-plates 16) and is monitored by microchips attached under the wells. In this way, the impedance difference is translated into a parameter known as the Cell Index (CI). Therefore, the higher the CI, the greater the number of cells adhered to the bottom of the well (Pinzon-Herrera et al. 2020). Based on our previous study, the results indicate two phases in the cell behavior: cells adhesion and cell proliferation phases, after 30 hours and between 30-72 hours of culture, respectively (Pinzon-Herrera et al. 2020).

Figure 4 (C) shows the CI values as a function of the first 72 hours of culture for the 6 experimental conditions for donor 1. Donor 1 shows a slow cell adhesion stage in the evaluated period, and it reaches a maximum peak around 18 hours. CI values reached a maximum of 6 CI units. Compared to the uncoated sensor without the IFN-γ, cell adhesion has three times higher CI value on (HEP/PLL) + CL without the IFN-γ. In addition, there is no detectable cell adhesion on (HEP/PLL) which confirm our results in cell viability. Regarding the anti-proliferative effect of the IFN-γ on hMSCs, (HEP/PLL) + CL with and without the IFN-γ show to be efficient compared to the uncoated surfaces. This finding indicates that the (HEP/PLL) + CL do not negatively affect hMSCs growth.

**Intracellular IDO Assay**

Indoleamine 2,3-dioxygenase (IDO) is a cytosolic heme protein that is important for immuno-regulatory functions (Takikawa et al. 1988)(Mbongue et al. 2015). It can be determined by measuring the amino acid kynurenine (pg/cell) ), which is known to be a catalyzer to convert L-tryptophan to kynurenine (Takikawa et al. 1988) (Däubener et al. 1994). The ability of IFN-γ to induce IDO expression in hMSCs was compared on TCP, and (HEP/PLL) + CL with and without IFN-γ supplemented in the cell culture medium after 6 days. The results of (HEP/PLL) are not shown because of the cells adhesion limitation (based on the results from cell viability and cells adhesion). Results for IDO activity are summarized in Figure 4 (B), which shows that for donor 1, all surfaces with IFN-γ (including TCP, and (HEP/PLL) + CL) have approximately five times a higher level of the IDO activity among surfaces without IFN-γ. Donor 2 shows that the IDO activity on TCP+ IFN-γ increases by adding IFN-γ in cell medium compared to TCP (Supplementary Information Figure S1(C)). In addition, the IDO activity on (HEP/PLL) + CL with IFN-γ has a higher activity compared to the (HEP/PLL) + CL without IFN-γ. These results comply with study done by Kwee et al. indicating the IDO activity corelated with amount of IFN-γ (Brian J. Kwee, Johnny Lam, Adovi Akue, Mark A. KuKuruga, K. Zhang, Luo Gu 2021).

Regarding the (HEP/PLL) + CL (with and without IFN-γ), donor 1 and donor 2 both show a decrease in amount of IDO activity compared to the TCP and TCP + IFN-γ, respectively.

These finding may indicate that both donors show that using the (HEP/PLL) +CL does not affect the level of the IDO activity in reference to the same amount of the IDO expression for the TCP and (HEP/PLL) + CL without IFN-γ. These in vitro studies indicate that the level of the IDO depends on not only the different donor's response but also IFN-γ and the multilayers influence the IDO expression. This result is in line with the study done by Cifuentes et al. (Cifuentes et al. 2020) that showed the IFN-γ is the key regulator of the IDO activity on heparin/collagen multilayers.

**Cells differentiation assay**

The ability of hMSCs to differentiate into osteogenic and adipogenic lineages cells was induced by supplementing the growth media with differentiation media. The differentiation ability of hMSCs was evaluated to confirm the multipotentiality of hMSCs culturing on TCP, (HEP/PLL), and (HEP/PLL) + CL with and without IFN-γ supplemented in the cell culture medium. After 10 days of incubation, cell functions associated with osteoblast differentiation (ALP activity,

calcium deposition) and adipogenic differentiation were evaluated. Mineralization was also characterized from microscope images. The impact of presence of the IFN-γ in the cell medium was also evaluated.

Figure 5 (A) and Figure 6 shows that there are areas visible with red and purple, indicating the formation of the calcified regions and adipocyte-like cells, respectively. Figure 5 (A) shows that cells on (HEP/PLL) expressed no staining due to lack of cells adhesion on (HEP/PLL). However, hMSCs cultured on TCP, and (HEP/PLL) + CL shows an increase in the size of calcium deposits formed by the clustering of cells due to the strong staining with Alizarin red, which indicates osteogenic differentiation of cells. The same results were found for donor 2, as shown in Supplementary Information Figure S2 (A). Also, hMSCs on TCP, and (HEP/PLL) + CL has the ability to differentiate to adipogenic cells, which the cells changed from long spindle-shaped to flattened round, or polygonal cells as shown in Figure 6 and Supplementary Information Figure S2 (B). In addition, Figure 5 (A) & Figure 6 show that the treatment with IFN-γ had no inhibitory effect on both the osteogenic and adipogenic differentiation of hMSCs. No staining was observed on cells cultured in regular expansion medium, as shown in Supplementary Information Figure S3.

ALP is an enzyme present in bone-related cells and is considered key to mineralization (Zhu et al. 2002). Its activity is related to the level of inorganic phosphate, a component of the bone mineral phase (Renata Francielle Bombaldi de Souza, Fernanda Carla Bombaldi de Souza, Andrea Thorpe, Diego Mantovani, Ketul C. Popat 2019). Therefore, ALP activity has been considered as an early indicator of osteoblast differentiation. Results for ALP activity are summarized in Figure 5 (B)Error! Reference source not found.. Also, ALP activity of undifferentiation cells is considered as controls. Regarding donor 1, (HEP/PLL) + CL with and without IFN-γ showed enhanced intracellular levels of ALP as compared to TCP. Also, the TCP and (HEP/PLL) + CL multilayer surfaces with IFN-γ have a higher ALP activity than the surfaces without IFN-γ. Similarly, donor 2 shows that (HEP/PLL) + CL without IFN-γ has a slightly higher ALP activity compared to TCP. However, (HEP/PLL) + CL with IFN-γ shows the same ALP activity compared to TCP, and less activity compared to the (HEP/PLL) + CL without IFN-γ Supplementary Information Figure S2 (C). Also, TCP with IFN-γ supplemented on cells medium shows a higher ALP activity compared to the TCP without IFN-γ. These in

vitro studies indicate that IFN-γ can improve the intercellular level of ALP activity. Also, the study done by C. Lamoury et al. indicated that IFN-γ resulted in affecting osteogenic differentiation of both mouse and human MSCs (Croitoru-Lamoury et al. 2011). As well, the study done by V. Semenov et al. (Semenov et al. 2009) show that crosslinking multilayers improve the cells differentiation compared to the non-crosslinked multilayers. Furthermore, these findings show that the differentiation of hMSCs may affected by donor's behavior, so differentiation of hMSCs need more studies.

The undifferentiated hMSCs controls (cultured in hMSCs growth medium) displayed no staining. However, cells show good differentiation on TCP and (HEP/PLL) + CL not only without IFN-γ but also with IFN-γ. These can indicate that the appearance of the IFN-γ and crosslinked multilayers do not suppress cells differentiation.

## Conclusion

This study demonstrates that polyelectrolyte multilayers made of heparin and poly(L-lysine) were successfully built up using the layer-by-layer assembly method. QCM, XPS, and Azure A results demonstrate the construction of the multilayers, and the changes it undergoes after chemical crosslinking. Also, this study evaluated the effect of crosslinked (HEP/PLL) multilayers on the growth and immunosuppressive properties of hMSCs. It shows that (HEP/PLL) + CL have a better cells growth, adhesion, proliferation, differentiation, and immunomodulatory properties compared to the (HEP/PLL). In addition, the (HEP/PLL) + CL show better cell viability compared to the tissue plastic culture even in presence of IFN-γ. However, (HEP/PLL) + CL show less immunomodulatory properties. In contrast to our previous study, we noticed that heparin/collagen multilayers have great stimulation on the protein expression, immunomodulator factor expression, and adhesion of hMSCs compared to the issue plastic culture. We believe that tissue culture plastic reduces the therapeutic potential of hMSCs due to the lack of extracellular matrix components. Furthermore, ECM components effect the cellular immunomodulator factor, differentiation, and growth. In the future, we will investigate the use of (HEP/PLL) + CL coatings to have a better understanding of the modulatory response

of hMSCs to soluble factors, which may improve hMSCs-based therapies aimed at treating several immune diseases and the cell manufacturing process.


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## Statement of Ethics

This work did not need approval from an ethic committee.

## Conflict of Interest

The authors have no conflicts of interest to declare.


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## Author Contributions

**Mahsa Haseli:** The LbL multilayers, XPS, QCM-D, Cell Culture, Cells viability Real-time monitoring of hMSCs, Immunomodulatory factor expression, Differentiation, experiments, Data analyses, Data curation and writing-original draft preparation, Figure preparation.

**Luis Pinzon-Herrera:** Qualitative colorimetric determination of heparin and data curation.

**Jorge Almodovar:** Conceptualization, supervision, writing-reviewing and editing, resources, funding acquisition, project administration.

## Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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## Figure Legends

Figure. 1. Absorbance for Azure A dye solution applied to TCP, (HEP/PLL) multilayers, and (HEP/PLL) + CL multilayers.

Figure. 2. QCM-D data showing the normalized frequency shift & dissipation shift as a function of time for the 3rd, 5th, and 7th overtones during the construction of the HEP/PLL multilayers with IFN-γ, with alternating 3-minute rinse and 5 minutes adsorption intervals. A&B: shows the normalized frequency shift. C&D: shows the normalized dissipation shift. Note that we use -Δf for a clear representation of the results.

Figure. 3. Chemical properties of the multilayers of (HEP/PLL) as measured by The XPS broad spectra and high-resolution XPS. (A): XPS survey scan spectrum of (HEP/PLL) and (HEP/PLL) + CL multilayers. (B): the corresponding specific spectrum of elemental (HEP/PLL) and (HEP/PLL) + CL multilayers.

Figure. 4. (A): PrestoBlue Viability assay for cultured hMSCs donor 1. Cellular behavior in cell cultures on TCP, (HEP/PLL), and (HEP/PLL) + CL multilayers with and without IFN-γ. (B): Real-time monitoring of hMSCs grown on TCP, (HEP/PLL), and (HEP/PLL) + CL multilayers after 72 hours with and without IFN-γ. (C): Cells immunomodulatory potential by IDO activity for hMSCs as a measure of picograms of kynurenine produced by cells cultured on TCP, (HEP/PLL), and (HEP/PLL) + CL multilayers with and without IFN-γ.(D): Fluorescence microscopy images of hMSCs nuclei and actin cytoskeleton, labeled with Hoechst and Actin Red, of cells attached to the TCP, (HEP/PLL), and (HEP/PLL) + CL multilayers with and without IFN-γ. Data are presented as the mean ± standard deviation of n = 4 samples. The p-values < 0.05 are represented by *, p-values < 0. 01 by **, p-values < 0. 001 by *** and p-values < 0.0001 by ****.

Figure. 5. hMSCs differentiation donor 1. (A): Osteogenic differentiations were stained by Alizarin Red. (B): Alkaline phosphatase (ALP) assays were performed after of induced osteogenesis on TCP and (HEP/PLL) + CL multilayers. Data are presented as the mean ± standard deviation of n = 4 samples. The p-values < 0.05 are represented by *, p-values < 0. 01 by **, p-values < 0. 001 by *** and p-values < 0.0001 by ****.

Figure. 6. hMSCs differentiation donor 1. Adipogenic differentiation were stained by Oil Red.

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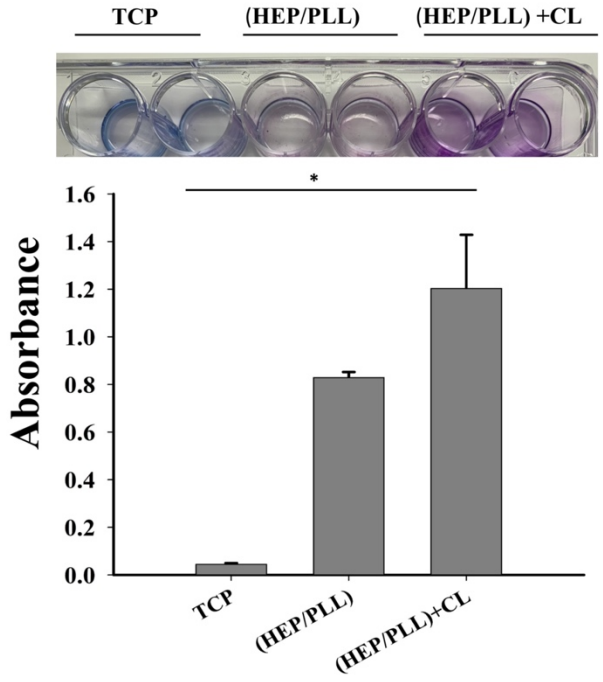
735 Figure 1

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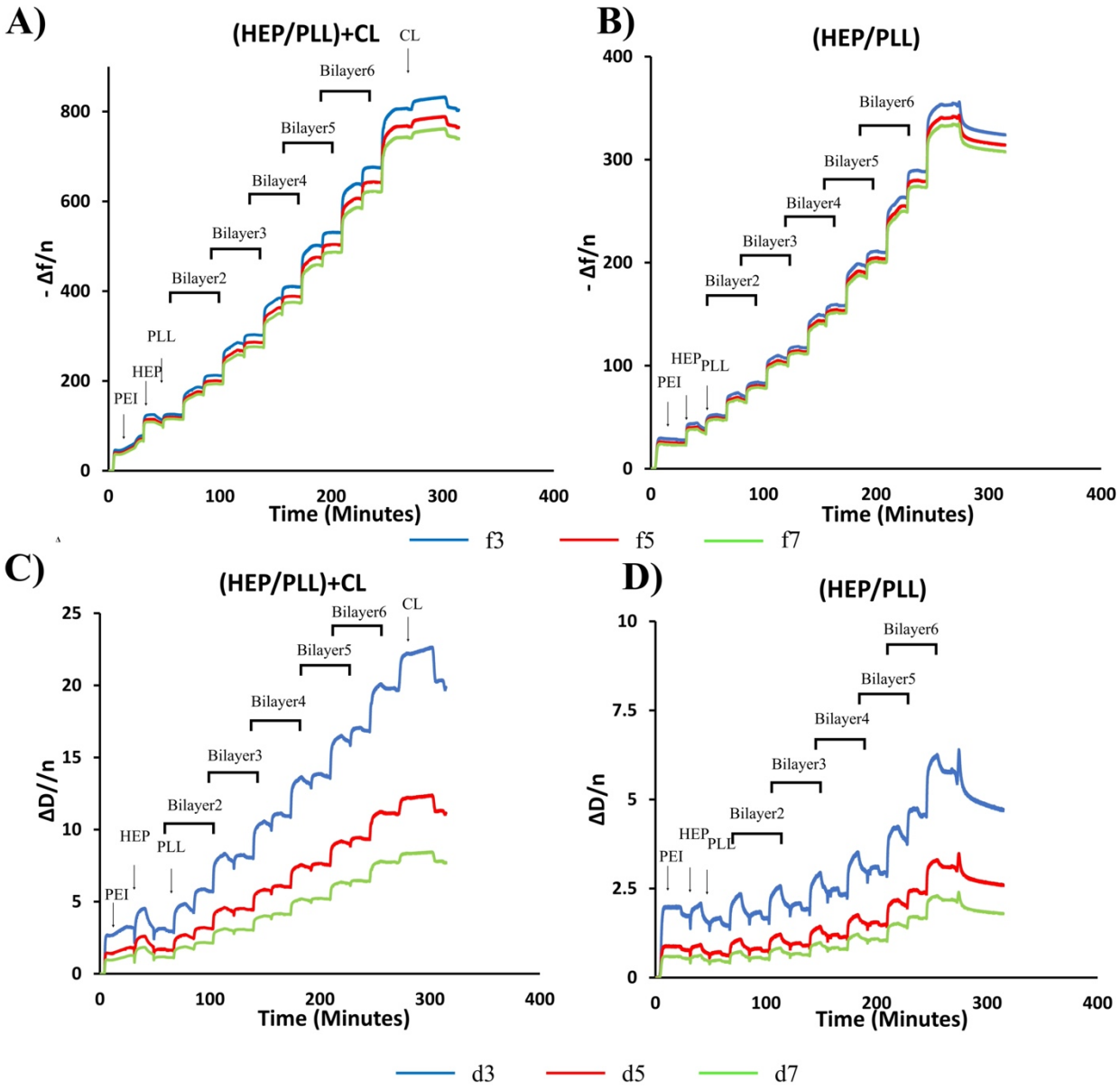
738 Figure 2

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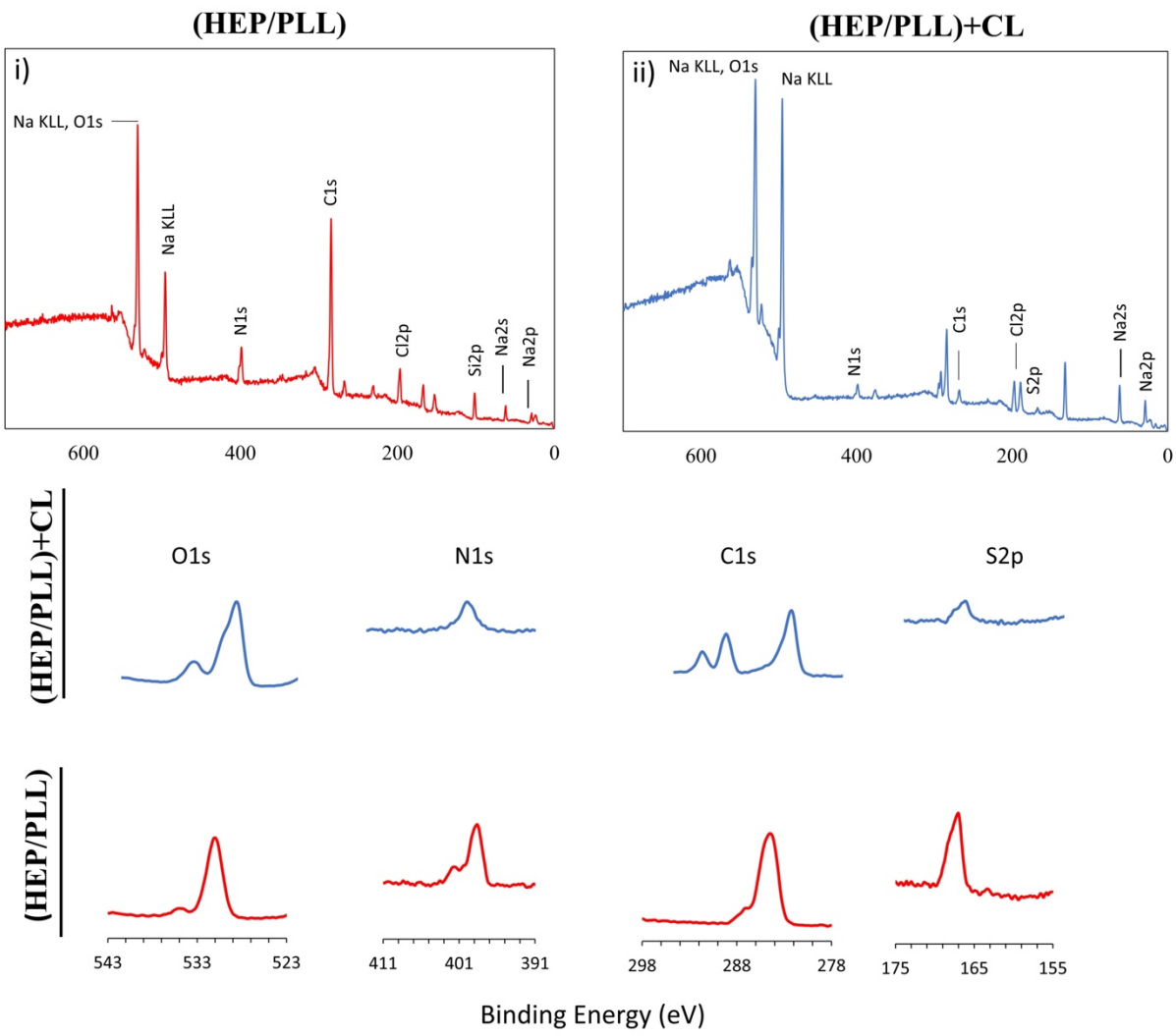
741 Figure 3

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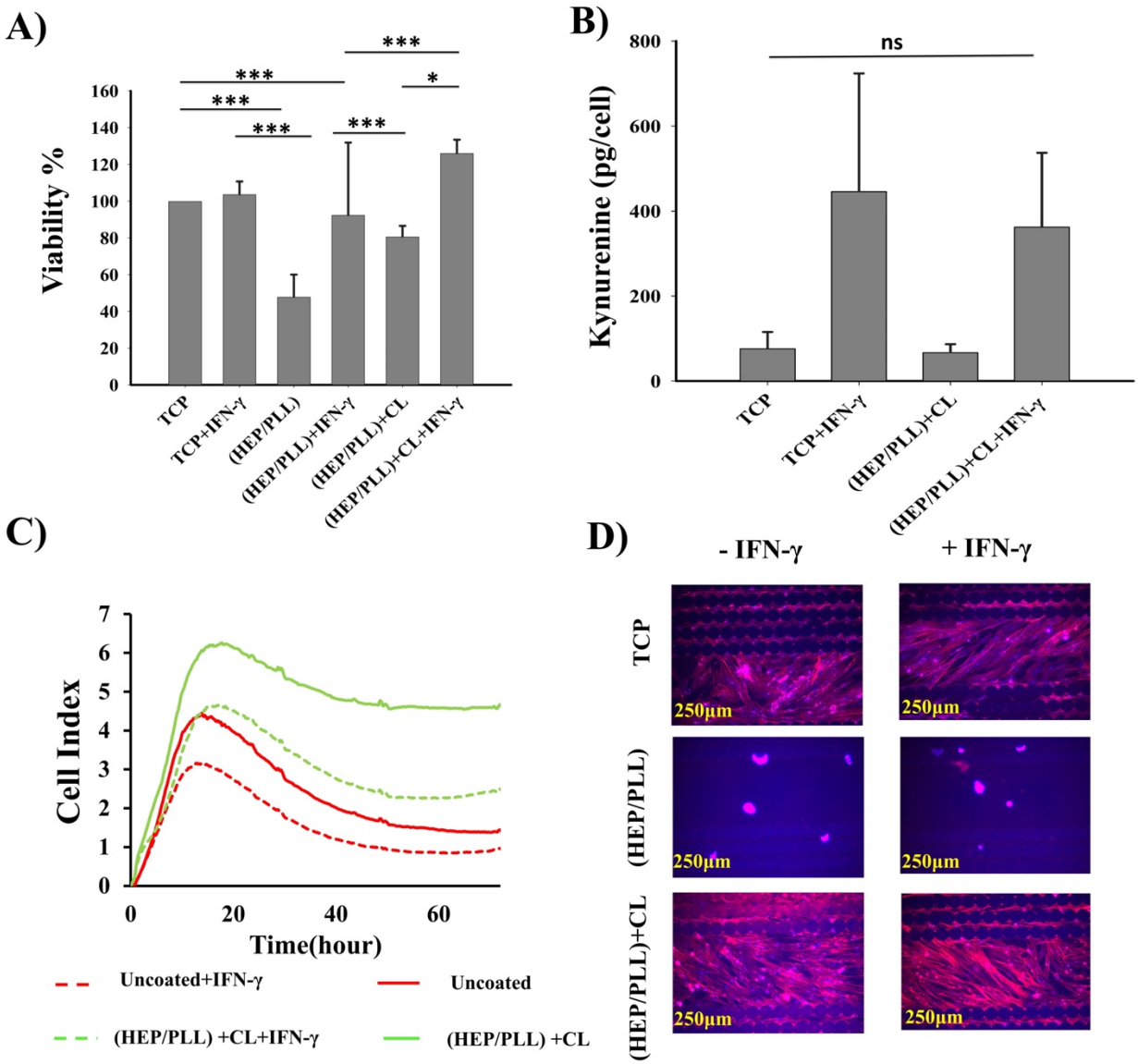
744 Figure 4

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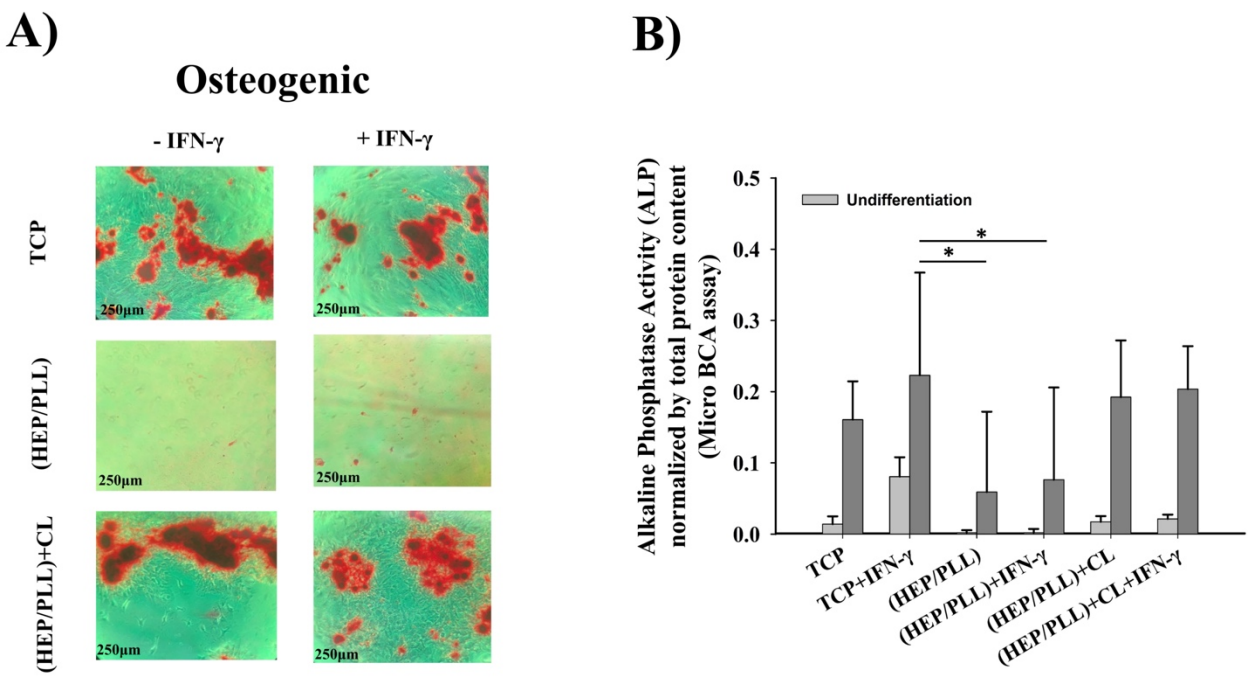
747 Figure 5

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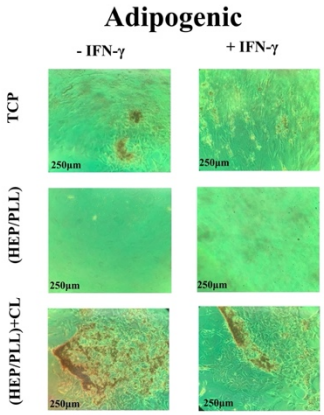
750 Figure 6

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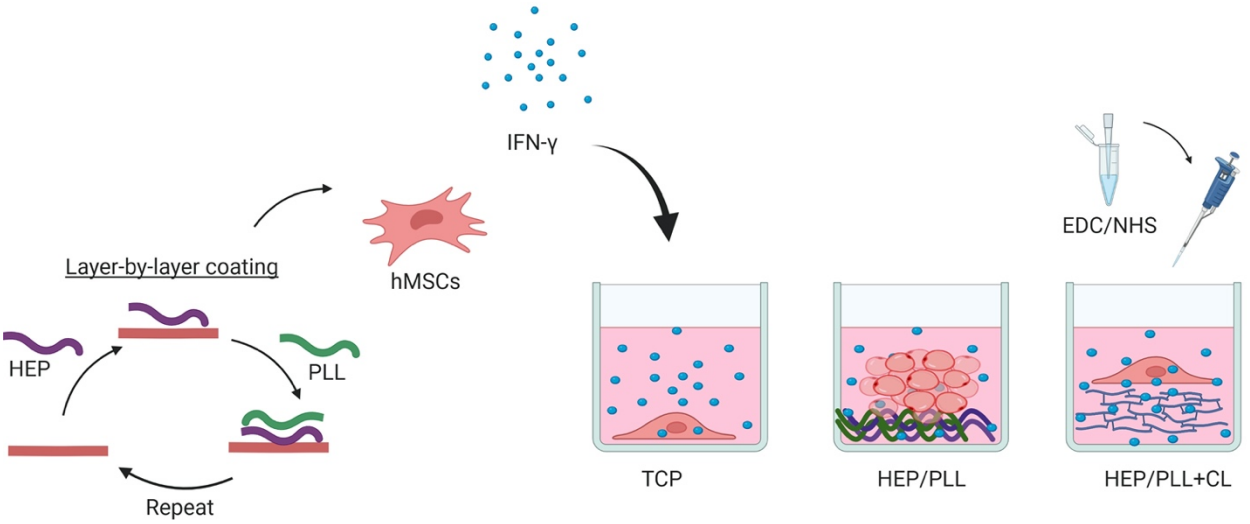
753 Graphical Abstract

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