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Study of the Stability of Heparin/Collagen Layer-By-Layer Coatings

An Undergraduate Honors College Thesis in the Department of Chemical Engineering

College of Engineering

University of Arkansas

Fayetteville, AR

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ABSTRACT

Pairing heparin with collagen-based medical implants has opened a whole new area of research for enhancing the desired effect of current implants. In fact, heparin (HEP) and collagen (COL) layer-by-layer (LbL) coatings have shown impressive results in forming polyelectrolyte multilayers. It has been already seen on skin grafts, nerve guide conduits (NGCs), and drug delivery devices yielding promising results. Due to being a simple, cost-efficient, and versatile option to fabricate thin biomimetic films, this self-assembly technique is one of the most effective methods to immobilize extracellular matrix (collagen and heparin) onto medical devices and implants. Even though previous studies have shown that HEP/COL coatings improve cell adhesion, migration, proliferation, and expansion of human Schwann cells (hSCs), the stability of these polymer coatings over time remains uncertain. Schwann cells are neuronal glial cells essential for maintaining the integrity, growth, and – important to our research - the regeneration of nervous tissue. This research focused on studying the stability of six bilayers of HEP/COL LbL coatings, which will be noticed as $(HEP/COL)_6$, when incubated in PBS (Phosphate Buffered Saline) and cell culture media. That is, how the $(HEP/COL)_6$ coatings degrade over a certain period and how cell behavior may be affected by the degradation level. The experiments monitored cell behavior in pre-treated coatings in real time and with a PrestoBlue viability assay. It was found that although the cell culture media treatment of the coatings initially offered better conditions to enhance cell behavior, it also rapidly deteriorated the coatings. Furthermore, it was observed that $(HEP/COL)_6$ favors the cell behavior even over three weeks.
Keywords: *heparin, collagen, layer-by-layer, Schwann cells, coatings, cell behavior, degradation*.

1. INTRODUCTION

Biopolymers are large molecules synthesized by the human body but can also be produced from synthetic material. Polymers are made up of smaller molecules, which are linked to form chains, these small molecules are called monomers. The use of biopolymers is quite wide, with outstanding benefits such as medical applications and drug delivery applications. Collagen is one of the biopolymers widely used, especially in coating surfaces for cell culture. Regardless of its limiting mechanical properties, collagen's biocompatibility and unique self-assembly make the introduction of crosslinking a better approach to producing collagen-based biomaterials with remarkably enhanced physical and chemical properties [1]. Another biomaterial utilized to a great extent in biomedical applications is heparin. Heparin's molecular structure makes it a great antithrombotic, and in fact, has been used for more than 70 years [2]. Much like collagen, heparin has significant chemical and physical properties that enhance Mammalian cell production.

Heparin and collagen are both very well-studied and understood biopolymers. Due to their nature and presence in the human body, they are optimal for mimicking in-vitro human body conditions. Researchers have proved that when heparin is introduced to collagen implants it greatly enhances the desired effect. Such applications can be observed in nerve guide conduits, drug delivery devices, and skin grafts [3]. Previous research has shown that proliferation, viability, and adhesion of human Schwann cells (hSCs) cultured on $(HEP/COL)_6$ are enhanced [4].

The stability of the coatings over time remained to be studied. The experiments outlined in this work monitored cell behavior in pre-treated coatings in real time and with a PrestoBlue dye

test. It was also studied the release of heparin from the coatings when submerged in cell culture media and phosphate-buffered saline (PBS).

2. METHODS

a. (HEP/COL)$_6$ Fabrication

Lyophilized type I collagen sponges from Integra Lifesciences Holdings Corporation, Anasco, PR; sodium heparin from Celsius Laboratories Inc. in Montevideo, UY (Cat. #PH3005); and poly- (ethylene imine) (PEI) from Sigma-Aldrich in St. Louis, MO (Cat. #P3143) were prepared with a sodium acetate buffer to fabricate the coatings in the wells of microplates. The sodium acetate buffer was prepared at two different pH levels (pH 5 and 4) with deionized water from a MilliporeSigma™ Direct-Q™ 3 in Burlington, MA (Cat. #ZRQSVP3US) at 18.2 MO cm, glacial acetic acid, and sodium acetate. The collagen was prepared with a pH 4 buffer while the heparin and PEI were prepared with a pH 5 buffer. The pH 5 buffer alone was also used as a rinse. The process is illustrated in Figure 1. Briefly, the method began by submerging the substrate with the PEI solution to form an anchoring layer for 15 minutes, followed by a rinse for 3 minutes. The heparin solution is then added and allowed to be set for 5 minutes, followed by another 3-minute rinse, 5 minutes for the collagen solution, and one more rinse, thus producing one bilayer of HEP/COL. The process is repeated the number of times needed to complete six bilayers. Finally, the coatings are briefly rinsed with 200 µL of deionized water and then sterilized under UV light for 10 minutes before cell culture.

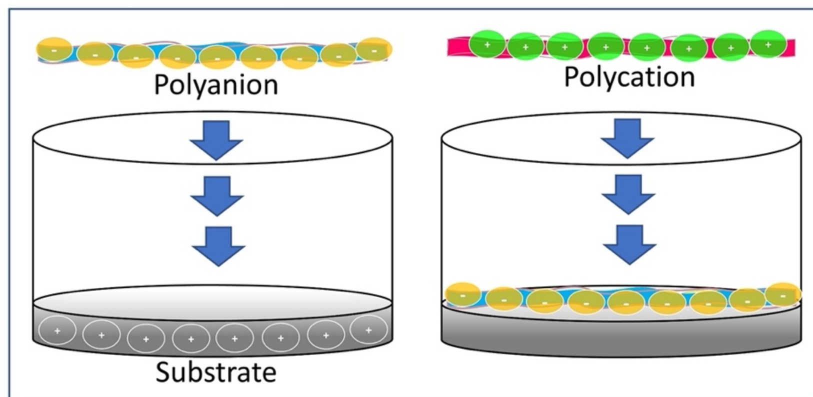


Figure 1. Layer-by-Layer Technique in a Well.

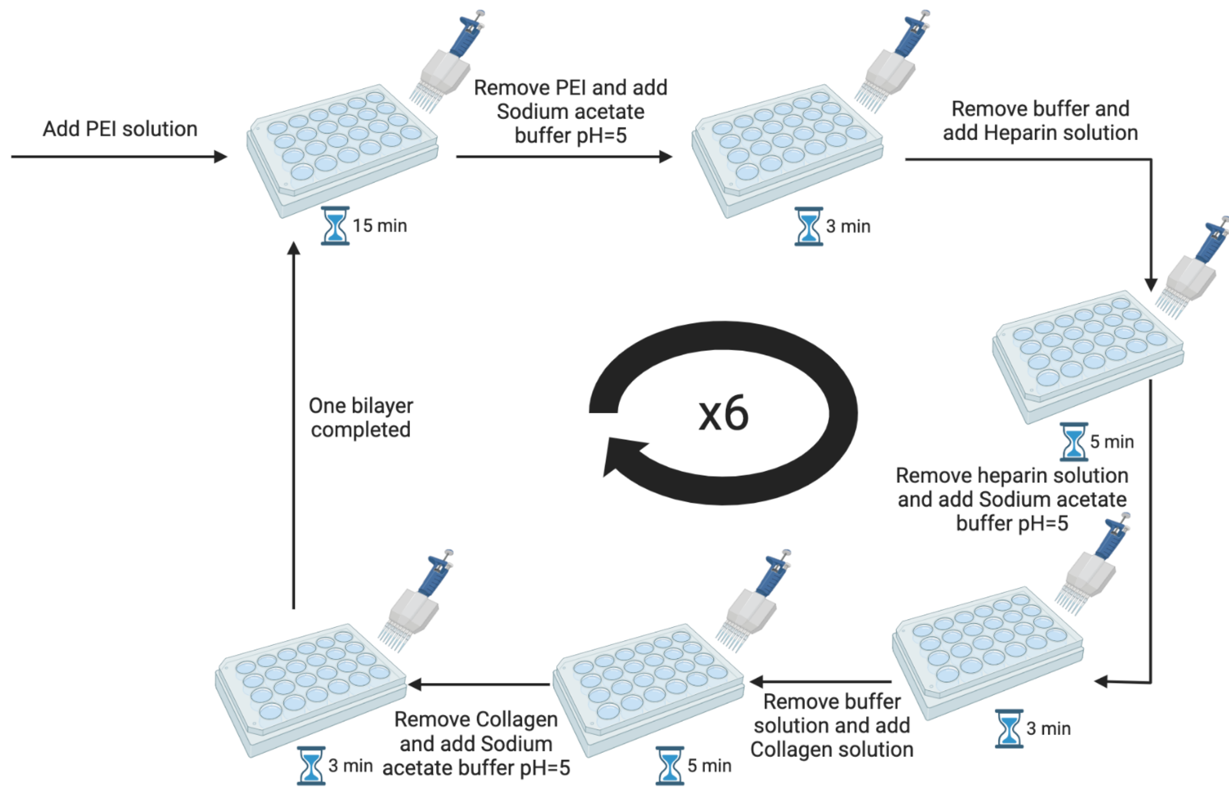


Figure 2. Layer-by-Layer Technique Applied to Cell Culturing Plates [5].

b. Cell Culturing

A cell culture media was prepared with Dulbecco's Modified Eagle's Medium from Sigma-Aldrich's (Cat. #D5648), sodium bicarbonate and sodium pyruvate, 1% Penicillin–streptomycin from Sigma-Aldrich's (Cat. #P4333), and 10% Fetal bovine serum from Gibco in Jenks, OK (Cat. #10-437-028). The cells were then incubated at 37 ºC with a 5% CO_2 air atmosphere. This cell culture media was used for the culturing of hSCs from ATCC in Manassas, VA (sNF96.2 ATCCs CRL2884t). Cells from passages 16 to 22 were used in experiments testing cell culture.

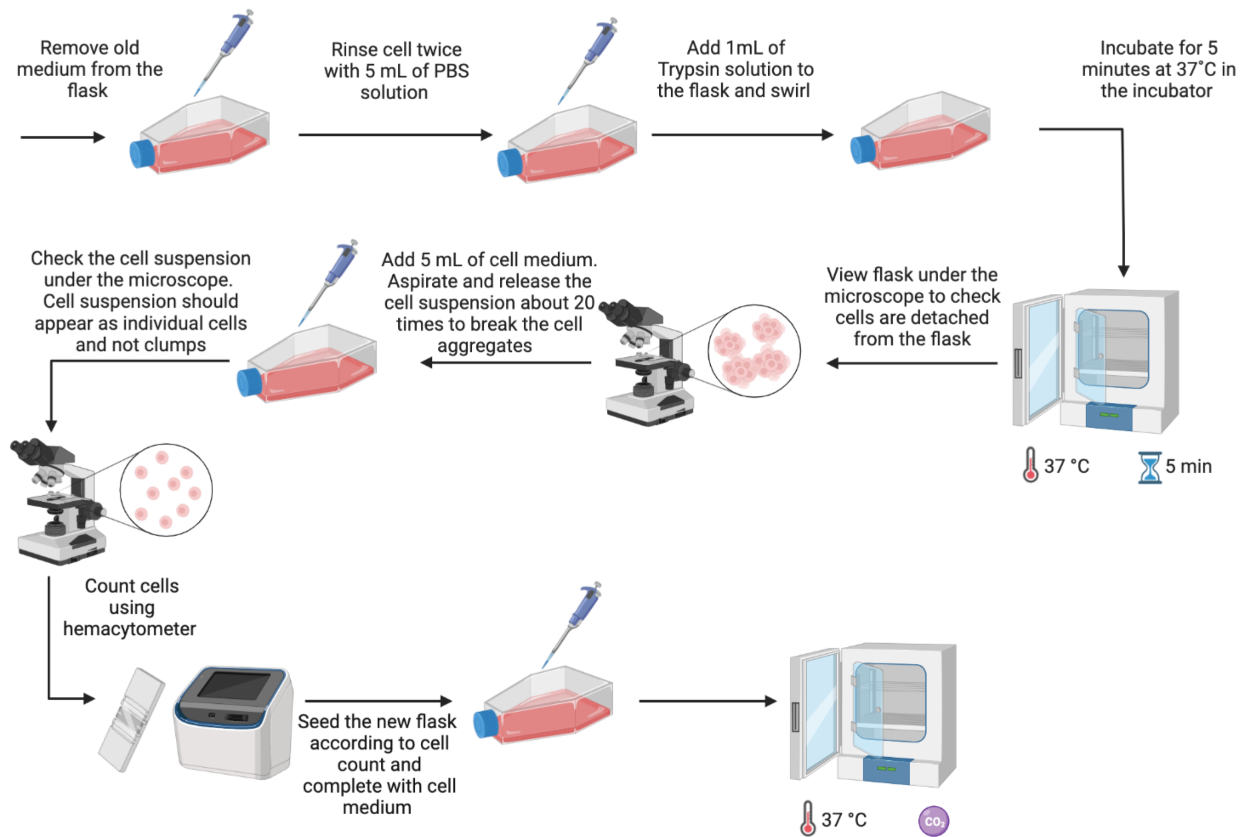


Figure 3. Cell Passage Procedure Performed with Schwann Cells [5].

c. Experimental Conditions

Experiments involving cell culture will be tested for six conditions:

- Cell culture media and cell culture media with Nerve growth factor (NGF), as the controls,
- $(HEP/COL)_6$ and $(HEP/COL)_6$ and with NGF, for plates with previous incubation with cell culture media; and,
- $(HEP/COL)_6$ and $(HEP/COL)_6$ and with NGF, for plates with previous incubation with PBS. The nerve growth factor (NGF) was used at 10 μg/mL dissolved in the cell culture media.

d. Pre-treatment of the Coatings and Plate Schedule

In experiments involving cell culture, each plate was tested at different times (0 days and 1, 2, 3, and 4 weeks). Each plate was incubated with the six previously described conditions for the time specified below per plate. On the day the plate was to be tested the supernatant of the pre-treatment would be removed, the cells cultured on the coatings, and the plate incubated again for six days. The cells were observed for the first three days.

- Week 0: 45 minutes with cell culture media or PBS
- Week 1: 1 week with cell culture media or PBS
- Week 2: 2 weeks with cell culture media or PBS
- Week 3: 3 weeks with cell culture media or PBS
- Week 4: 4 weeks with cell culture media or PBS

e. PrestoBlue Cell Viability Test

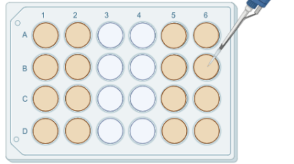
Ten Corning™ 3603 96-well clear bottom black polystyrene microplates (Fisher Cat. #07-200-565) were prepared with $(HEP/COL)_6$ in 16 wells each. Five of the plates were incubated with cell culture media and 5 with PBS from 0 days, and 1, 2, 3, and 4 weeks. Cells were cultured on one plate each week under the six conditions described above. After 3 days of cell culture, the supernatant was removed, and we added to each well 100 µL of a mixture containing 90:10 of cell culture media and PrestoBlue™ cell viability reagent from Invitrogen (Cat. #A13261). After 3 hours of incubation, the plate was read in the BioTek Multi-Mode Microplate Reader (Model Synergy™ 2).

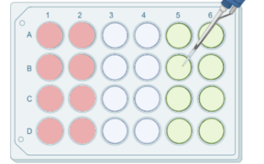
f. Real-time Monitoring of Cell Adhesion

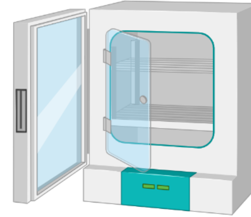
We prepared ten 16-well E-Plates 16 (Cat. #300600840 from ACEA™) with $(HEP/COL)_6$ in 12 wells each. Five of the plates were incubated with cell culture media and 5 with PBS from 0 days, and 1, 2, 3, and 4 weeks. Cells were cultured on one plate each week under the six conditions described above. After cell culture, the plates were then monitored in real-time for three days using xCELLigence RTCA S16. The device was placed inside the incubator and allowed to warm up, taking care to clean any condensation.

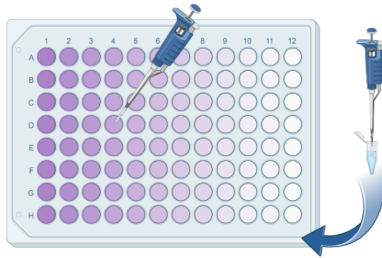
g. Heparin Release Study

One 24-Well Corning™ Costar™ flat-bottom polystyrene microplates (Fisher Cat. #07- 200-740) were prepared with coatings in 8 wells each. The plate had coatings submerged in 1mL of cell culture media, as well as with 1 mL of phosphate-buffered saline (PBS 1x) from Thermo Scientific™ (Cat. #28372). The plate was then incubated for 30 days, and every 72 hours samples of the supernatant were taken. The volume was replenished with their respective reagent and once again incubated. 50 μL of each sample was then deposited in a Corning™ 3603 96-well clear bottom black polystyrene microplate (Fisher Cat. #07-200-565) and the content of heparin was determined using the Azure A blue dye colorimetric method by measuring the absorbance using a BioTek Multi-Mode Microplate Reader (Model Synergy™ 2) at 620 nm.









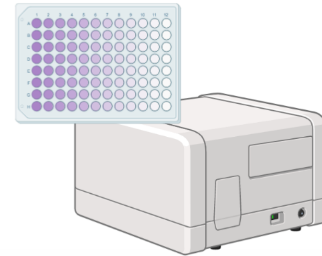


Figure 4. Azure A Blue Dye Colorimetric Method [5].

3. RESULTS AND DISCUSSION

PrestoBlue Cell Viability Test

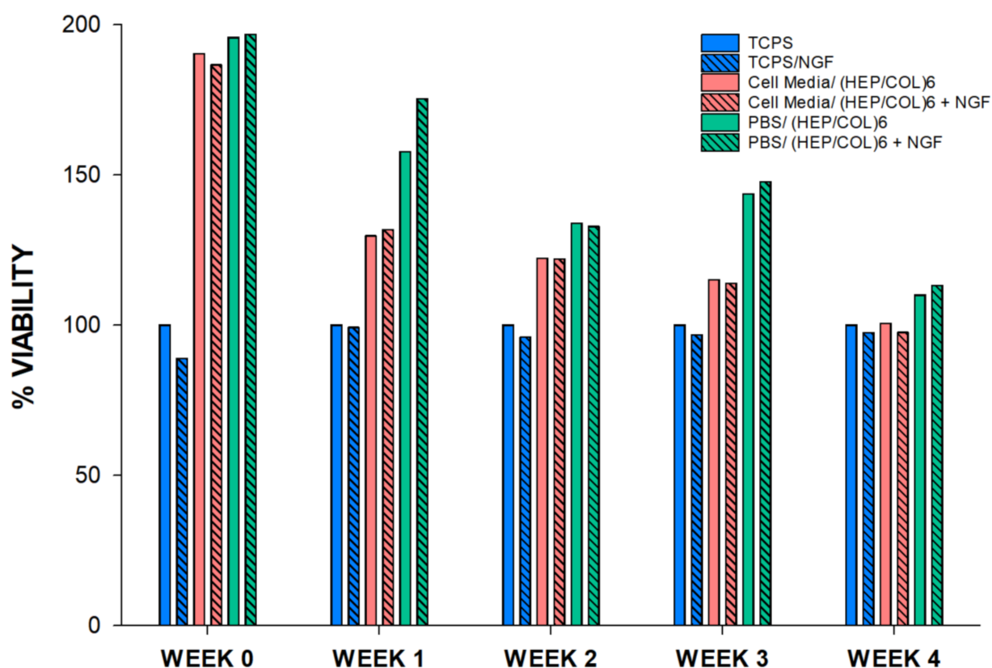


Figure 5. Cell Viability Over Four Weeks.

The results of these assays aid in the further study of the number of viable cells in the well plates. With the help of the plate reader, results from Week 0 to Week 4 can be analyzed, and review what trends follow. This test shows how viable the cells are over a period of 4 weeks by analyzing the marker activity associated with cell number. Figure 5 displays the percentage of viable cells nearly doubling when cultured on $(HEP/COL)_6$ surfaces in comparison with the TCPS condition in the plate denominated as week 0. After 1 week had passed, the potency of the cell culture media treated coatings greatly decreases. Even though the cells treated with cell culture media decrease after 1 week, this can still produce some viable cells up until week 3. As can be observed, the change from week 1 up until week 3 is not a dramatic one, it follows more of a steady trend. The PBS-treated coatings have a steady decline in viable cells found after initial cultivation. From week 1 to week 2, the change displayed remarkably decreases, while it

can be seen that this one increases in week 3. At week 4, there no longer seems to be an improvement in cell viability in coatings. The TCPS condition shows similar results as the ones treated with cell culture media and PBS.

xCELLigence Cell Adhesion Monitoring

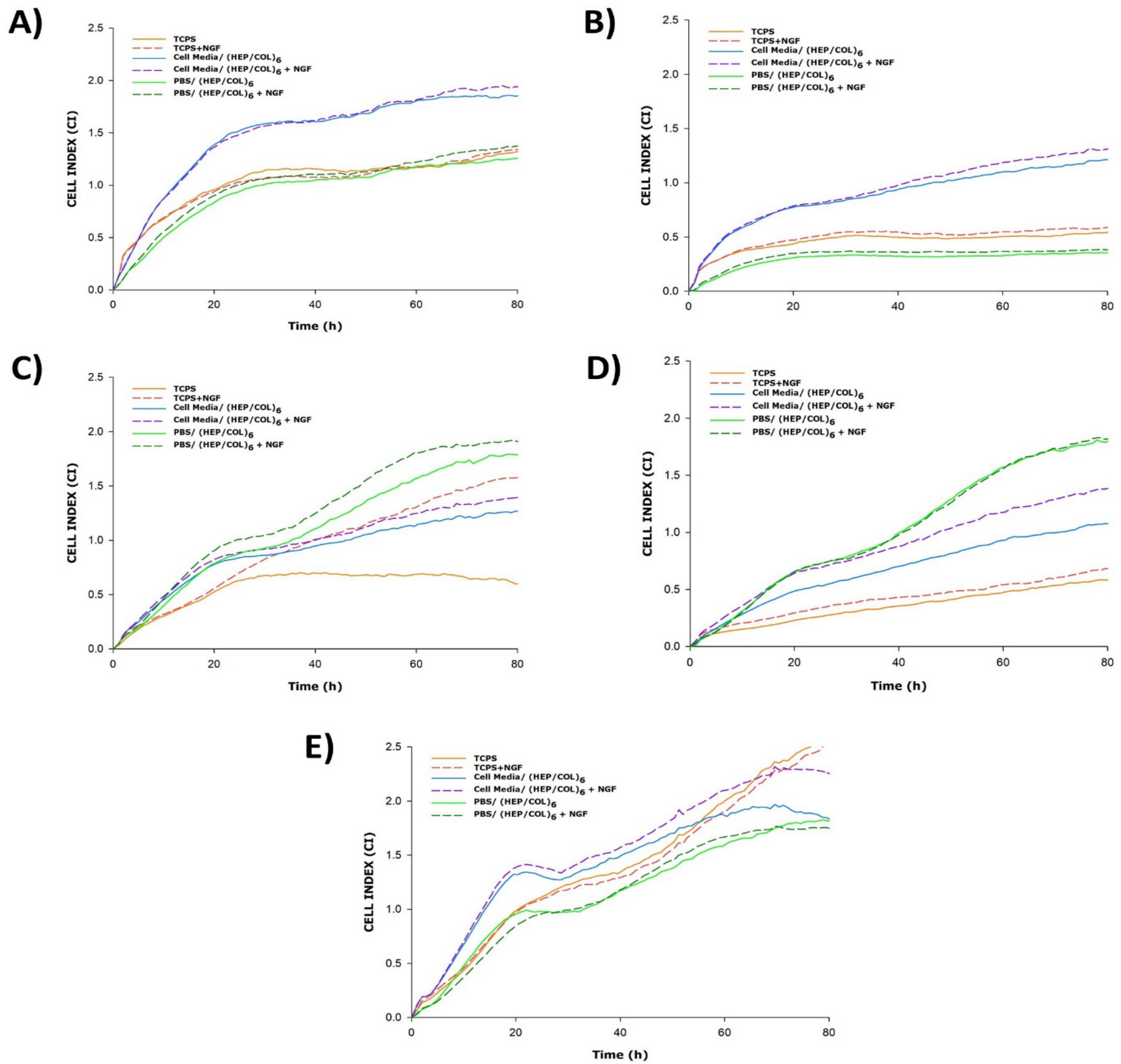


Figure 6. Real Time Monitoring for A) Week 0, B) Week 1, C) Week 2, D) Week 3, and E) Week 4.

Real-time cell behavior was analyzed by using the RTCA iCELLigence which allows measuring the proliferation of the cell using biosensors. This technology makes possible the further study of cell migration, profitability, growth, and viability. This is accomplished by measuring the bioelectrical impedance utilizing microchips found in the microplates. The results obtained are graphed by taking the Cell Index, which is taken as a proliferation, growth, and adhesion measurement, and plotting it against time (hours).

For weeks 0 and 1 (Fig. 6A and 6B) the cells adhere better in the cell culture media conditioned coatings. In figures 6A and 6B, the higher cell index readings were found in the coatings treated with cell media, especially the coating treated with cell culture media and NGF showed a higher cell index than the ones treated only with cell culture media. The nutrients in the cell culture media may be coupling with the coatings, encouraging cell adhesion. However, the potency of the cell culture media condition begins to rapidly drop after week 1, since in weeks 2 and 3 (Fig. 6C and 6D), the PBS treated coatings offer less degradation than the cell culture media. In this case, it can also be seen that the coatings treated with PBS and NGF showed a better cell index than the ones treated just with PBS. Interestingly, at week 2 (Fig. 6C), the TCPS+NGF showed a better cell index in the coatings treated with cell culture media. At week 4 (Fig. 6E), the coatings may be for the most part degraded, regardless of the cell culture media or PBS treatments.

Heparin release study

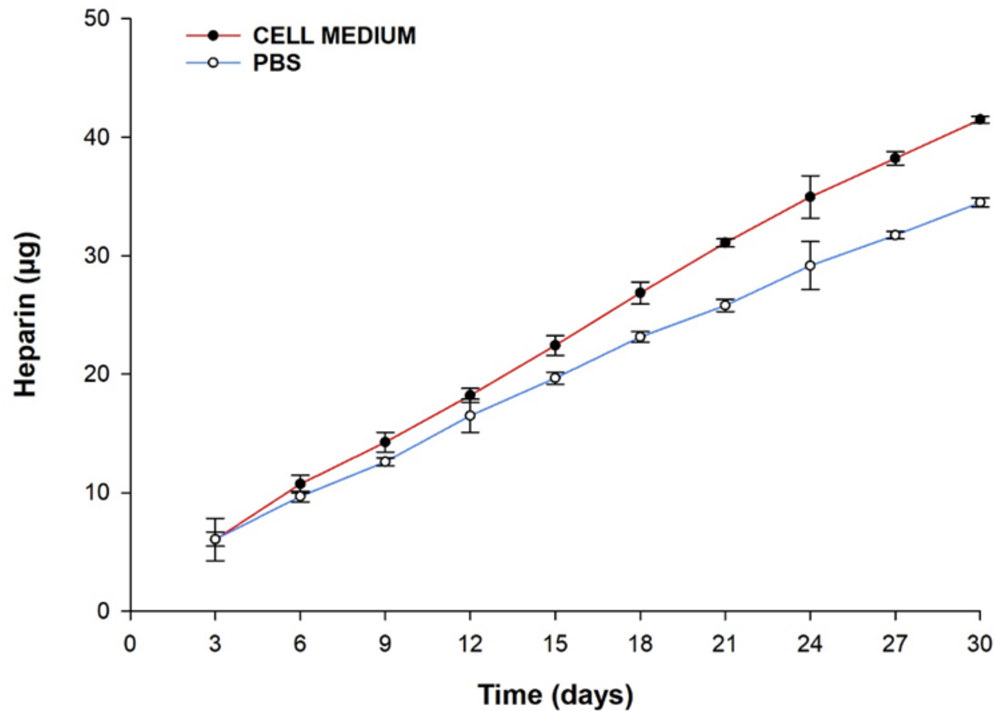


Figure 7. Heparin Release of Treated Wells with Cell Culture Medium and PBS.

In the heparin study release conducted, it can be observed in Fig. 7 that the coatings submerged in cell culture media released more heparin than the ones submerged in PBS within the first 72 hours. This helps to understand why cells have a much-reduced response to the culture media after the first week of the cell viability and adhesion tests. The heparin release shows a linear increment in both cell culture media and PBS over 30 days. Figure 7 shows how the heparin is being released at an increasing linear rate every 72 hours. While the PBS treated coatings also show an increasing heparin release over the experiment time, it does happen in a lower quantity than the cell culture media treated coatings.

4. CONCLUSIONS

This work provides evidence that the $(HEP/COL)_6$ on TCPS does not compromise the integrity of the surface and human Schwann cells. $(HEP/COL)_6$ stability can be evidenced for at least 3 weeks since the results of the cell viability and adhesion experiments in week 4 did not show a significant difference between the culture conditions. It can also be concluded that coatings pre-treatment with the cell culture media for a week seems to favor improved behavior of the cells; however, after 2 weeks the cell culture media seems to greatly destabilize of the coating compared to those pre-treated with PBS. Moreover, cell culture media demands a greater effect of degradation of $(HEP/COL)_6$, in terms of heparin release, in comparison with the PBS.

5. ACKNOWLEDGEMENTS

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