CELL PROLIFERATION AND MIGRATION ASSAY ON POLYMER SURFACES

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Abstract. Cell proliferation and migration on phosphonated polymer surfaces may impair cell adherence and migration. The aim of the present study was to evaluate the ability of osteoblast-like cells to migrate at the surface of the phosphonated chitosan pellicle compared to normal chitosan pellicle. Results. Cells proliferated and migrated at different rates according to surface charge. Negative surfaces showed lower adherence and the migration difference could be quantified. Conclusions. Cell migration on polymer surfaces can be successfully evaluated by improved scratch test performed directly on polymer pellicles. Migration assay can be quantified by measuring the scratch size following separator removal. At the same time, viability assays are applicable following this improved scratch test while with no cells are damaged by mechanical means mechanically damage.

INTRODUCTION

Cell proliferation and migration on polymer surfaces may quantify not only survival rate but also cell adherence according to various surface properties (Bhattacharyya, et al., 2010, Choi, et al., 2012, Guidoin, et al., 2005). Polymer phosphorylation by phosphorous acid, due to extensive negative charges determined by phosphite groups, may impair cell adherence and migration.

One of the most abundant natural biopolymer is represented by chitin which is extracted from crustaceans, fungal cell walls and insects (Francesko and Tzanov, 2011, Jayakumar, et al., 2011, Mir, et al., 2008). This natural biopolymer can be deacetylated to obtain chitosan. Together with other natural polymers, as polyvinyl alcohol and cellulose, chitosan is part of a new class of biomaterials with various functions due to their biological activity, excellent biocompatibility, and complete biodegradability together with low toxicity (Jayakumar, et al., 2008, Wang and Liu, 2014).

As chitosan represents a main element in osteoconductive and drug delivery systems and while chitosan functionalization by phosphorylation may play an important role in cell migration in various scaffolds used in regenerative medicine, the aim of the present study was to evaluate the ability of osteoblast-like cells to migrate at the surface of the phosphorylated chitosan pellicle compared to normal chitosan pellicle.

MATERIAL AND METHODS

Chistosan blends and membranes

1% chitosan solution was obtained by chitosan powder (Fluka) dissolution in 1% aqueous acetic acid solution at room temperature. Chitosan pellicles were obtained by pipetting 0.2 mL of chitosan solution on 96-well tissue culture plate, and then by drying in a dry incubator at 40°C for 48h.

2% collagen solution was added to chitosan solved in 1% acetic acid, to improve cell adherence. Differences in cell adherence were observed between simple chitosan solution and phosphorylated chitosan.

Prior to cell incubation, coated plates were sterilized by UV action in a transilluminator for 30 minutes. Following UV sterilization, each well was washed three times by sterile PBS then dried again in the dry incubator, in sterile conditions.

Cell cultures

For the present study, MNNG-human osteosarcoma cell line – HOS (CLS, Germany) was used. These cells have a similar phenotypic profile with osteoblast cells and are usually used in most assays regarding biocompatibility, cell proliferation and viability in contact with polymer or metallic biomaterials.

Complete medium used was composed by alpha MEM supplemented by 10% FCS, 1% Glutamin and 1% Antibiotic/antimycotic (penicillin /streptomycin). Cells at passage 5 were thawed for 90 sec at 37°C in a waterbath, washed in 10 ml complete culture media and centrifuged for 5 min at 400 G in a 15 ml centrifuge tube. The media was removed and the pellet resuspended in 1 ml warm fresh complete media. Cells were counted and viability evaluated by a Countess system using trypan blue (Invitrogen). Cell count showed values of $1x10^6$ with 80% viability. Resuspended cells were distributed in two 75 cm^2 flasks with 20 ml fresh warm medium each. Cells were placed in an incubator at 37°C with 5%CO_2. Cell subconfluence was obtained at 2 days from the initial passage. Cells were washed by PBS to remove traits of FCS and then detached by 1x TrypLE (Invitrogen). Cells were subsequently dispersed in 96 well plate, with the well’s bottom coated by chitosan-collagen and phosphorylated chitosan-collagen pellicles, and with separators glued to the plate lid (figure 1).

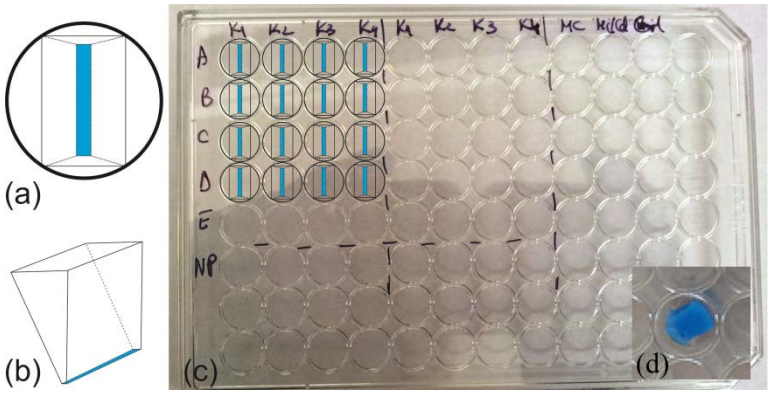

Figure 1. Silicone separator attached to a 96 well plate lid to simulate scratch test without impairing polymer surface. (a) top-view; (b) side-view; (c) 96-well plate lid with glued separators (draw); (d) Silicone separator attached to the lid of a 96 well plate

Prior to lid placement, each well was filled by 70µl complete media with $1x10^5$cells. Plates were incubated for 24 h for the cells to allow attachment and proliferation, and then the lid with separators was removed and replaced by a new one, with no separators. Plates were incubated again for another 48 hours to allow cells proliferate on the polymer pellicle side that was partially covered by the separator on the bottom of each well.

Plates were imaged at 24, 48 and 72 h for 3D cultures with a Nikon D-5000 camera adapted by a C-tube to a Nikon T-3000 reversed phase contrast microscope.

Viability assay - MTT

Cell viability by MTT assay (Mosmann, 1983) is a versatile method used to evaluate the cell survival following incubation with extraction liquid (LEx) from the investigated samples. It is a colorimetric method that uses a tetrazolium salt (MTT) which is transformed by mitochondrial dehydrogenases in purple formazan granules. MTT is a yellow hydrosoluble powder that can be metabolized by active (viable) cells, generating the formazan granules that can be subsequently dissolved by DMSO. Results are interpreted on spectrophotometric reading plates at 570-590 nm

Following medium removal, cells in 24, 48 and 72 wells were incubated with MTT solution (1 mg/ml in PBS) for 3 h and the resulting formazan was dissolved by DMSO (100 µl). Absorbance was measured at 590 nm using an automated multiplate reader (Pharmacia LKB Ultrospec Plus). Cell viability was expressed as percent compared to control lanes (blank - culture medium without cells; control – culture medium with cells) according to the formula CV=100x (ODs-ODb)/(ODc-ODb), where ODs represent the optical density (in units) for the sample, ODb – the optical density for the blank wells and and ODc – the optical density for the control wells. Assays for each extract were carried out in three replicates, including untreated cell control and the blank cell-free control.

RESULTS

Following cell spreading in the 96 well plates, in each well at 24 hours, there were observed aspects of cell proliferation, with an improved better spreading and adherence for the blend containing chitosan and collagen and compared with a lower adherence and proliferation rate for to the phosphorylated chitosan and collagen blend (figure 2). The starting point for simulated scratch filling was at 24 hours from initial cell spreading into the wells. Thus, further observations were performed at 48 and 72 hours respectively. At 48h, cells spread on chitosan-collagen membrane had a more obvious trend to close the scratch than on the phosphorylated polymer surface. Supplementary negative charges at the phosphorylated chitosan membrane surface influences also cell proliferation and adhesion. Thus, it appears that a relationship could be observed between lower surface adherence on such polymer blends and cell proliferation. Results are more obvious at 72 hours, cell proliferation producing wound closure at this

incubation time for the simple blend. At the same time, on the phosphorylated polymers surface, cell proliferation was somehow poor, with reduced cell density and lack of real confluence. The simulated wound was not closed completely at 72 hours for the phosphorylated chitosan-collagen blend (figure 2). While visual results and wound healing measurements were reproducible but gave no indication related to cell viability, an MTT test was performed in a second 96-well plate, incubated at the same time with that submitted to simulated scratch test.

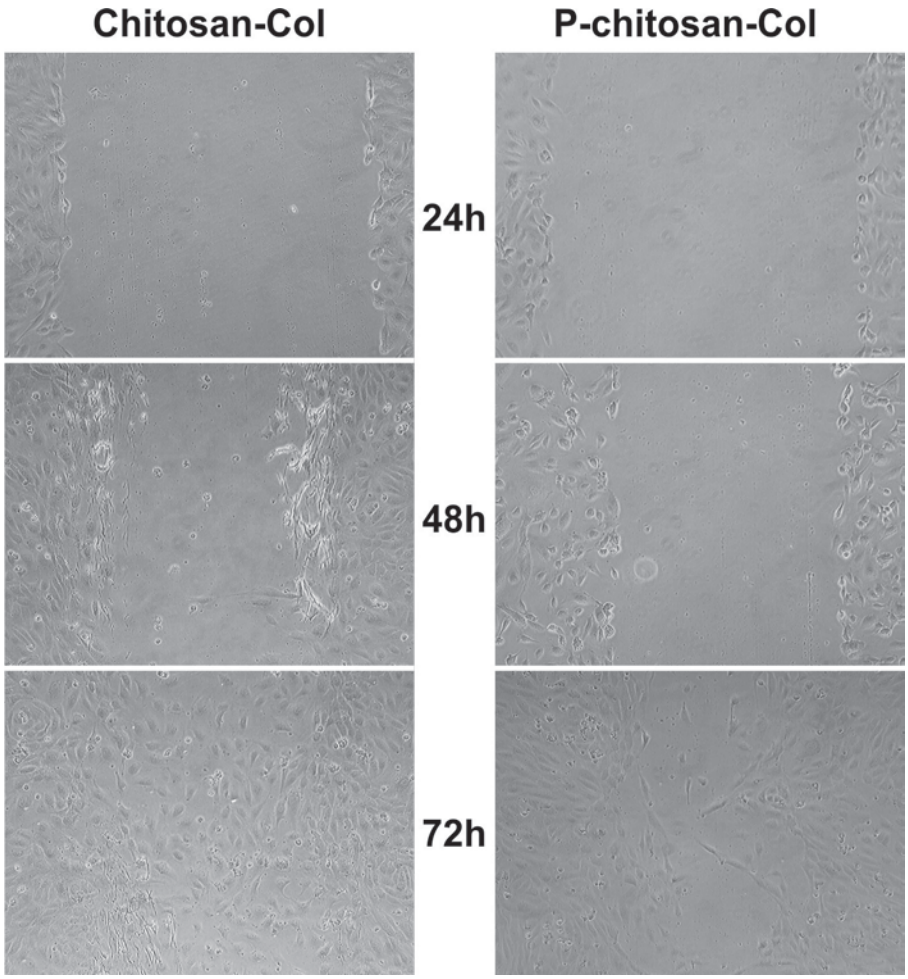

Figure 2. Scratch test using silicon separator on 2 chitosan membranes (left – chitosan-collagen and right phosphorylated chitosan-collagen)

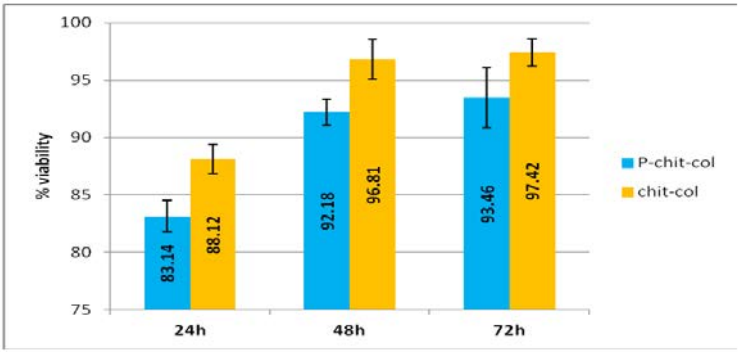

Figure 3. HOS cell viability at different times following dispersion on chitosan and chitosan-phosphate-collagen blend membranes. Error bars – 95% confidence interval.

At 24 hours, viability was over 80% for cells proliferating on both membranes (figure 3). Cell viability improves at 48 an 72 hours but the difference between simple chitosan-collagen and phosphorylated chitosan-collagen membranes are still visible. We may interpret this result as a consequence of lower adherence of the phosphorylated polymer membrane on cell viability and proliferation rate. However, cell viability was good overall, and in order to appreciate polymer surface effects on cell behavior, one should explore integrin expression or other adhesion proteins presence at cell-polymer interface and also caspase activity in proliferating cells.

CONCLUSIONS

Cell migration on polymer surfaces can be successfully evaluated by improved scratch test performed directly on polymer pellicles. Customized scratch test on polymer surface with no pellicle/membrane impairment is a cheap and feasible procedure to evaluate cell behavior on sensitive interfaces (as cell polymer one). Simulated scratch test allows the evaluation of cell adhesion, proliferation and survival at various polymer interfaces. Considering that cell viability remained very good following this assay, we may also recommend it as a preliminary test regarding adhesion proteins expression on various polymer surfaces following scratch assay. Also cells from this test may be further fixed and stained or submitted to DNA/RNA extraction for molecular analysis of adhesion protein expression or caspase activity (flow cytometry).

REFERENCES

Bhattacharyya, D., Xu, H., Deshmukh, R. R., Timmons, R. B., & Nguyen, K. T. (2010): *Surface chemistry and polymer film thickness effects on endothelial cell adhesion and proliferation*. J Biomed Mater Res A, 94(2), 640-648.

Choi, J., Konno, T., Takai, M., & Ishihara, K. (2012): *Regulation of cell proliferation by multi-layered phospholipid polymer hydrogel coatings through controlled release of paclitaxel*. Biomaterials, 33(3), 954-961.

Francesko, A., & Tzanov, T. (2011): *Chitin, chitosan and derivatives for wound healing and tissue engineering*. Adv Biochem Eng Biotechnol, 125, 1-27.

Guidoin, M. F., Guidoin, R., Frayssinet, P., Legrand, A. P., & How, T. (2005): *Poly-L-lactide surfaces subjected to long-term cell cultures: cell proliferation and polymer degradation*. Artif Cells Blood Substit Immobil Biotechnol, 33(4), 411-422.

Jayakumar, R., Prabaharan, M., Sudheesh Kumar, P. T., Nair, S. V., & Tamura, H. (2011): *Biomaterials based on chitin and chitosan in wound dressing applications*. Biotechnol Adv, 29(3), 322-337.

Jayakumar, R., Selvamurugan, N., Nair, S. V., Tokura, S., & Tamura, H. (2008): *Preparative methods of phosphorylated chitin and chitosan--an overview*. Int J Biol Macromol, 43(3), 221-225.
Mir, V. G., Heinamaki, J., Antikainen, O., Revoredo, O. B., Colarte, A. I., Nieto, O. M., & Yliruusi, J. (2008): *Direct compression properties of chitin and chitosan*. Eur J Pharm Biopharm, 69(3), 964-968.
Mosmann, T. (1983): *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays*. J Immunol Methods, 65(1-2), 55-63.
Wang, K., & Liu, Q. (2014): *Chemical structure analyses of phosphorylated chitosan*. Carbohydr Res, 386, 48-56.
Mosmann, T. (1983): *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays*. J Immunol Methods 65(1-2): 55-63.
Wang, K. and Q. Liu (2014): *Chemical structure analyses of phosphorylated chitosan*. Carbohydr Res 386: 48-56.

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