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RESEARCH ARTICLE / ARAŞTIRA MAKALESİ

Macro-Capsule Fabrication via 3D Printing for Mesenchymal Stem Cell Encapsulation

Mehmet Ali KARACA [1], Derya Dilek KANCAĞI [2], Uğur ÖZBEK [3], Ercüment OVALI [2], Özgül GÖK [4]

[1]Department of Medical Biotechnology, Institute of Health Sciences, Acibadem Mehmet Ali Aydinlar University, Istanbul, Turkey

[2]Acibadem Labcell Cellular Therapy Laboratory, Istanbul, Turkey

[3] Medical Genetics Department, School of Medicine, Acibadem Mehmet Ali Aydinlar University, Istanbul, Turkey

[4] Department of Medical Engineering, Engineering and Natural Sciences Faculty, Acibadem Mehmet Ali Aydinlar University, Istanbul, Turkey

Abstract
Cell transplantation is a widely used method to induce cell-mediated immune reactions inside the body. However, possible immune responses to the transplanted cells decrease the efficiency of applied cell therapy. This issue can be addressed by the transplantation of cells via 3 Dimensional (3D)-printed polymeric capsules which encapsulate cells and protect them from immune system attacks. Cell-loaded capsules (macro or micro) have emerged as potential carriers for more efficacious cellular therapies. In this study, 3D-printed porous capsules were prepared from biodegradable and biocompatible polyester "polycaprolactone (PCL)" and this macro-capsule was evaluated as a carrier for its cell encapsulation effectiveness. The macro-capsule was designed to have dimensions of 2x5x10 mm and drawn in Autodesk Fusion 360 program. PCL was utilized for its 3D bio-printing via Axolotl Bioprinter Dual Print Head System. The leakage structure on the macro-capsule was visually controlled by surface electron microscopy (SEM). The permeability of the macro-capsule was tested with human serum albumin (HSA) protein and trypan blue dye. Sterilization of the obtained macro-capsule was achieved via Ultra Viole (UV) light and the cytotoxicity of the polycaprolactone capsule was tested for 24 and 72 hours incubation periods. The semi-permeable macro-capsule was successfully obtained as a closed and hollow form. Its porous structure was demonstrated using trypan blue dye. To evaluate the porosity of the macrocapsule, human serum albumin (HSA) protein release was performed from the macrocapsule. It has been shown that 98% of HSA was released from the macrocapsule within 24 hours. The PCL macrocapsule was sterilized using UV light and was reported to show no *in vitro* cytotoxicity. In addition, it was shown that the cells in the macro-capsule consumed at least 10% glucose from the outside medium during 12 days of incubation, compared to 2-Dimensional (2D) cell culture conditions, and were able to release at least 8% of the lactic acid molecules outside. In conclusion, reproducible fabrication of polymer macro-capsule, high viability of encapsulated cells inside, and their metabolic assessment results have obviously indicated the potential of these capsules as effective carriers for living cells with transplantation-dependent cellular therapies.
Keywords: Macro-capsule, Polymeric Capsule, 3D Bio-printing, Cell therapy, Mesenchymal Stem Cells

Öz
Hücre nakli, vücutta hücre aracılı bağışıklık reaksiyonlarını indüklemek için yaygın olarak kullanılan bir yöntemdir. Bununla birlikte, nakledilen hücrelere karşı olası bağışıklık tepkileri, uygulanan hücre tedavisinin etkinliğini azaltır. Bu sorun, hücreleri enkapsüle eden ve onları bağışıklık sistemi saldırılarından koruyan 3 boyutlu (3B) baskılı polimerik kapsüller aracılığıyla hücrelerin nakli ile çözülebilir. Hücre yüklü kapsüller (makro veya mikro), daha etkili hücresel tedaviler için potansiyel taşıyıcılar olarak ortaya çıkmıştır. Bu çalışmada, biyobozunur ve biyouyumlu polyester "polikaprolakton (PKL)"den 3B baskılı gözenekli kapsüller hazırlanmış ve bu yarı-geçirgen makro-kapsüllerin hücre transplantasyonu için etkin bir taşıyıcı olup olmadığı incelenmiştir. Tasarlanan makro kapsül (2x5x10 mm) Autodesk Fusion 360 programında çizilmiş ve PKL materyali ile Axo Bioprinter Dual Print Head System kullanılarak basılmıştır. Makro-kapsülün kapalı formu üzerinde sızıntı, yüzey elektron mikroskobu ile görsel olarak kontrol edilmiştir. Makrokapsülün geçirgenliği tripan mavi boya ve ayrıca insan serum albümin (ISA) proteini ile test edilmiştir. Polikaprolakton malzemelerinin hücreler

Corresponding Author: Özgül GÖK, **Tel:** 0 216 500 4188-dahili, **e-posta:** ozgul.gok@acibadem.edu.tr
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üzerindeki sitotoksisitesi 24 ve 72 saatlik zaman dilimlerinde test edilmiştir. Elde edilen sonuçlara göre kapalı ve içi boş olacak şekilde yarı-geçirgen makro-kapsül formu başarı ile elde edildi. Makro-kapsülün gözenekli yapısının olduğu kapsülün tripan mavi boya kullanılarak gösterildi. Makrokapsülün gözenekliliğinin değerlendirilmesi için makro-kapsülden insan serum albümin protein salınımı yapıldı. ISA proteininin miktarca %98'inin 24 saat içinde makro kapsülden dışarıya salındığı gösterildi. PKL makro-kapsül UV ışığı kullanılarak sterilize edildi ve *in vitro* şartlarda sitotoksisite göstermediği raporlandı. Ayrıca makro-kapsül içindeki hücrelerin 12 günlük inkübasyon sırasında 2 boyutlu (2B) hücre kültürü şartlarına göre en az %10 oranında dışarıdaki besiyerden glikoz tüketimi yaptıkları ve ürettikleri laktik asit moleküllerinin en az %8'ini içerden dışarıya salabildikleri gösterildi. Polimer makro-kapsülün tekrarlanabilir üretimi, içerideki enkapsüle hücrelerin yüksek canlılık oranı ve bunların metabolik değerlendirme sonuçları, bu kapsüllerin transplantasyona bağlı hücresel terapilerde canlı hücreler için etkin taşıyıcılar olma potansiyelini açıkça göstermiştir.
Anahtar Kelimeler: Makro Enkapsülasyon, Polimerik Kapsül, 3B Biyo-yazıcı, Hücresel Tedavi, Mezenkimal Kök Hücre

I. INTRODUCTION

Cell therapy on tissue regeneration with patient-derived tissue or cell sources paves the way for new therapeutic opportunities for patients. Mesenchymal stem cell-based therapy is applied in various types of diseases and degenerative tissue such as tendons [1], intervertebral disk [2], bone [3], and articular cartilage [4]. Various cell types such as beta cells were used for the regulation of hormone and growth factor levels for the treatment of patients. Clinically islet (Beta cell) transplantation is an alternative way to exogenous insulin independence for prolonged periods. The main limitations of islet transplantation are the lack of organ donors, inconsistent islet yield, and multiple organ donors per patient [5]. The other severe limitation of the islet transplantation points out to the host immune rejection from recipient immune cells. These recipient immune cells and host immune rejection eliminate the long-term functionality and survival of the transplanted cells [6]. However, decreasing the survival rate of delivered cells inside the patient body directly correlated with a decrease in the efficiency of cell transplantation therapy. So, immunosuppressive drugs have emerged as alternative tools for obtaining effective cell therapy but some immunosuppressive such as glucocorticoids had significant islet toxicity [5]. Immunological problems in cell therapy resulted in the development of new therapeutic strategies for the long-term survival of transplanted cells. Encapsulation of the cells via biocompatible and semi-permeable macro-capsules is an attractive way for tissue regeneration. So, implantable, and retrievable macro-capsules for cell therapy seem to provide outstanding advantages to improve the therapeutic efficiency, especially in genetically engineered cell transplantation ranging from the elimination of frequent administrations of cells to the isolation of delivered cells from immunogenic attacks [7].

Polymeric capsule-based strategies have yielded promising results in various disease models without the need for immunosuppressing agents [8]. The cell-encapsulation strategy has emerged as a powerful tool for protecting cells from immunoreactions, hence allowing the nutrient transfer to maintain cell survival and function inside [9]. Encapsulation strategies as macro and micro size were applied to mammalian cells for different clinical usage [10]. Capsules have the potential to possess a special role as a barrier between transplanted cells and immune responses inside the body. Implantable and scalable macro-capsules were designed to obtain long-term viability and functioning of the delivered cell inside the patient body [10]. On the other side, large-sized macro-capsules which are 100.000 times smaller than a regular organ size might demonstrate limited organ function [11–13]. Still, studies on transplantation of islet cells loaded retrievable macro-sized capsule design showed that macro-capsule might provide long-term usage for diabetes in clinical applications [14–16].

Retrievable macro-capsule constructs have been utilized as attractive scaffolds for the delivery of various cell types and genetically modified cells to regulate protein or growth factor levels in protein deficiency diseases [17]. Especially, an immunosuppressive feature of mesenchymal stem cells (MSCs) was shown to be enhanced in terms of the efficiency of the islet transplantation via increasing nitric oxide production and secreting higher levels of immunomodulatory cytokines [17]. Based on these findings, the potential of MSCs for repairing damaged tissue, promoting angiogenesis, and reducing inflammation might be revealed to a greater extent for patient-specific cellular therapies. In this study, we have designed a semi-permeable 3D macro-capsule for the proper delivery of MSCs, which can be evaluated as a container for the encapsulation of cells with high cell viability. Although several biomaterial-based encapsulation techniques have been previously established for cell transplantation, this design provides a container-based reservoir of encapsulated cells with a more effective, cheaper, reproducible, and achievable strategy for further cellular therapies. A biocompatible and biodegradable PCL polymer was preferred for 3D printing of this macro-capsule, for which the level of porosity was analyzed by measuring the amounts of transported biomarkers throughout such as consumed glucose and produced lactic acid. MSCs with high viability up to 72 hours might contribute to the development of effective approaches for biomaterial-based cellular therapy.

II. MATERIALS VE METOD

2.1. Preparation of Macro-capsules

Designed macro-capsule were 3 dimensionally (3D) drawn in Autodesk Fusion 360 program. Drawn with assigned dimensions as 2x5x10 mm, macro-capsules were printed by using Axolotl Bioprinter Dual Print Head System (Axolotbio, Turkey) which was loaded with polycaprolactone (PCL) (Mwt: 80kDa). The highly permeable membrane through the capsule structure was expected to allow the transportation of nutrients and growth factors inside the capsule to the environment. The permeability of the porous membrane was optimized with various printing options (feed rate: 25%, 70 psi, first layer height: 2, extruder temperature: 120 °C, working stage: 55 °C, printing path: random). Printing options of programs (REPETIER-HOST and SLIC3R) change capsule structure and permeability of the porous membrane. Lastly, the macro-capsule is visually controlled to the presence of leakage on the closed form of the macro-capsule. Then, the permeability of the macro-capsule is tested via the transportation of human serum albumin (HSA) protein and trypan blue dye from inside to capsule to outside during the day.

2.2. Permeability Study

Obtained macro-capsules were checked for their permeability abilities by using Trypan blue dye as an indicator. Trypan blue dye (50µL) was injected into the macro-capsule. The injection zone was filled with melted polycaprolactone. Trypan blue release from microcapsule was analyzed after 1 day of incubation time.

2.3. Protein Release Profile

Human serum albumin (50 µL/ 10000 µg) (20% HSA, Octapharma, Switzerland) was used to test protein transportation between inside to capsule to outside during the day. Injection of HSA inside the capsule was sealed with melted PCL. Protein-loaded macro-capsule was periodically transferred to fresh test tubes containing an equal volume of fresh PBS solution in each period at RT (0, 0.5, 1, 3, 6,12, and 24 hours). Releasing of HSA content to the environment each time at RT was determined and quantified by using a calorimetric measurement BCA assay kit (BCA assay, Takara, Shiga, Japan) according to the manufacturing procedure.

2.4. Morphological Analysis

Macro-capsule was coated with 3.5 nm of gold-palladium and visualized with SEM (Thermo Fisher Scientific QuattroS, ABD). The imaging process was done under a low vacuum with an EDS detector at 5 kV.

2.5. Cell Culture Experiments

Green fluorescence particle (GFP) tagged human umbilical cord mesenchymal stem cells (Acıbadem Labcell, Turkey) were cultured in MSC nutrient-free medium (Biological Industries, Sartorius, Israel) and incubated at 37 °C with 5% CO_2. The waste medium was removed, and fresh medium was added every 48 hours until the 80% confluency of the flask was reached. Then, cells were trypsinized, centrifuged at 300 RCF for 10 minutes, and then collected in ringer lactate solution (containing 0.5% HSA by volume). Cell number and cell viability were counted with a cell counter device (TC20 Automated Cell Counter, BioRad, ABD).

Sterilization of macro-capsules was performed with serial washing with 10mM PBS and 4% (v/v) ethanol solution. UV sterilization procedure for 2 hours was applied after the antibiotic treatment (2% Penicillin-streptomycin) to the macro–capsules, which were later on directly and indirectly contacted to MSC cells for the measurement of their cytotoxicity effects. Prior to the experiment, cells were GFP-tagged so that cytotoxicity assessment of the cells was performed by comparing the GFP intensity signals (Image J program).

This process was conducted comparably for both cells in cryoprecipitate (Kızılay, Turkey) solution and non-cryoprecipitate solution encapsulated into the macro-capsules for 15 days of incubation time. Each group of cells (250.000 cells in 50µL media) was injected into the macro-capsules, and the area of the injection site was sealed with previously melted PCL polymer. Afterward, these macro-capsules were incubated in MSCs nutrient-free medium for 15 days at 37 °C and 5% CO_2. At different time points (1, 3, 5, 7, and 15 days), the culture medium was renewed and collected media was evaluated for the measurement of glucose consumption level and lactic acid production level. The collected cultivation medium was analyzed via the ADVIA® 1800 Clinical Chemistry System (Siemens, Germany) [18].

III. FINDINGS AND DISCUSSION

3.1. Findings

A 3D macro-capsule design was constructed via Autodesk Fusion 360 program and verified with the Repetier-host program (Figures 1 A, and B). This designed capsule was printed by using Axolotl Bioprinter Dual Print Head System (Figure 1C) with extrusion (pneumatic) based bioprinting, where a synthetic polyester polymer, PCL, was first melted at the syringe upon heating up to 120 °C, and then it was printed on the plate at room temperature with 0.25 mm nozzle. The permeability of the obtained macro-capsule was tested with 0.5% Trypan blue dye. It was observed that the aqueous part of trypan blue solution (prepared in 10mM PBS) was released into the environment, but the blue-colored dye stayed inside the capsule after 1 day of incubation at room

temperature. Compared with small molecular weight dye molecules, the permeability of the printed macro-capsules was also studied with HSA, as a model for high molecular weight, but the globular biological protein-based sample. As a time-dependent manner, almost all HSA injected into the macro-capsule's inner cavity was demonstrated to be released within 6 hours and 98% of total HSA protein was measured to be released from the macro-capsule in 24 hours of incubation (Figure 1D). Lastly, the sealed site of the leakage form for the porous capsule after the injection of the solution of interest inside was imaged after the completion of the release study under SEM microscopy. The injection site of the macro-capsule was successfully sealed with PCL and the injection site of the macro-capsule was stable after releasing the protein out as shown in Figure 1E. Moreover, a closed and hollow form of the PCL-based macro-capsule was used for cell culture experiments as a control group (Figure 1).

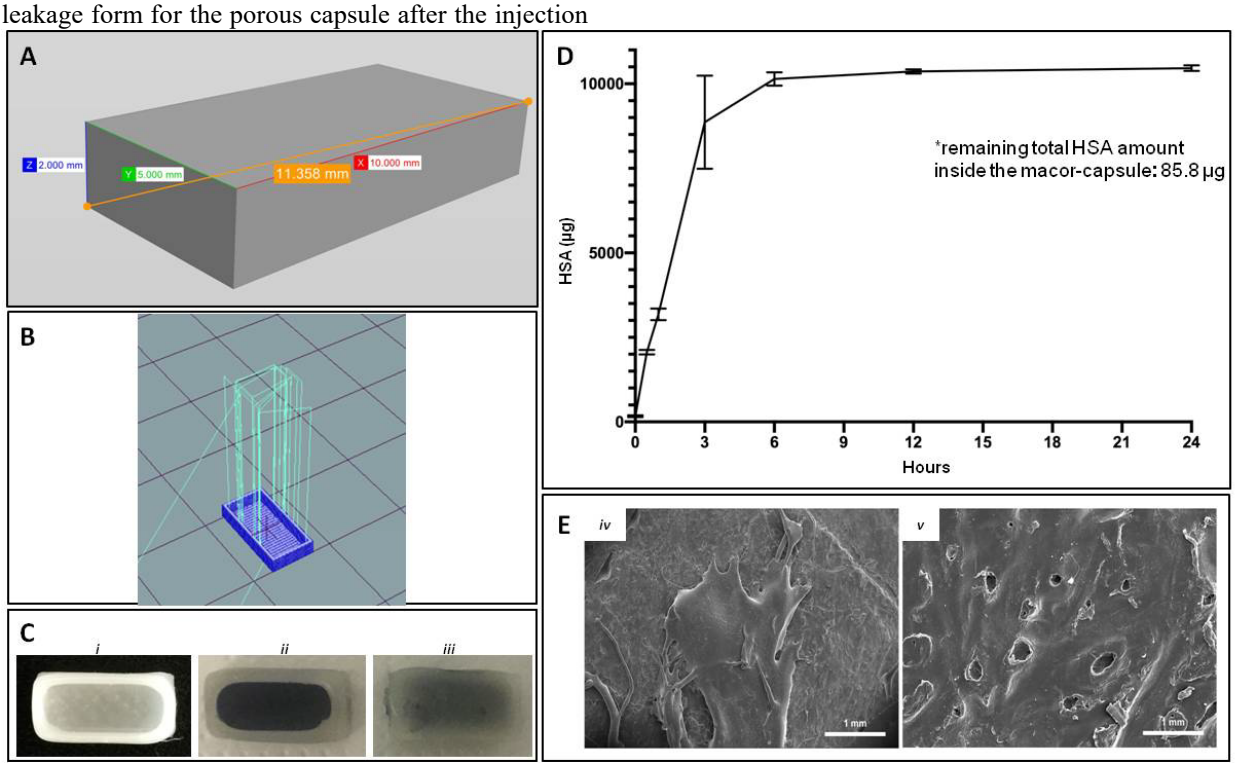

Figure 1. (A) Dimensions of designed macro-capsule with Autodesk Fusion 360 program, (B) printing path for 3D Bio-printer, (C) closed form of macro-capsule (*i*) trypan blue loaded macro-capsule (*ii*) closed form of trypan blue loaded macro-capsule (*iii*), (D) HSA release profile upon its diffusion from the macro-capsule (E) SEM images of the macro-capsule before (*iv*) and after (*v*) HSA permeability tests.

After sterilization of the macro-capsules, their cytotoxicity behavior was evaluated on GFP-tagged MSCs. Two different methods were applied to the macro-capsules for their interaction with cells. In the former one, macro-capsules were directly put on top of the seeded cells in a petri dish. On the other hand, the indirect contact method included the incubation of cells with a media where macro-capsules were previously incubated for 72 hours at 37 °C and 5% CO_2. After incubation of cells for 24, 48, and 72 hours, cells were visualized under fluorescent microscopy for their morphology as well as their GFP expressions as an indication of cytotoxicity level. Figure 2A compares the adhered cells on petri dishes with the non-treated MSCs as a negative control group. Significant cytotoxicity levels were not observed for MSCs investigated for their indirect interaction with the prepared macro-capsules. However, a slight decrease in the cell viability was detectable for the ones subjected to the direct contact method depending on the incubation time. This behavior was also confirmed by the measurement of the GFP fluorescent signal of processed images by the Image J program (Figure 2B). This difference in obtained cytotoxicity results may be attributed to the elimination of nutrient uptake by macrocapsule as incubation time gets prolonged. Additionally, it was observed that MSCs were detached from petri dishes upon their being scratched due to direct contact with the outer surface of the macro-capsule. However, cell attachment and proliferation were not observed under the macro-capsule contact surface.

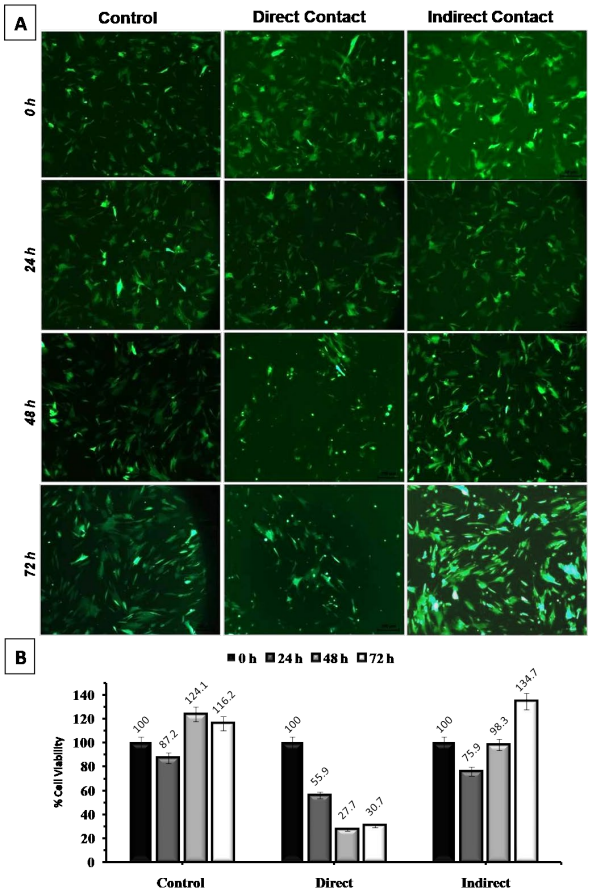

Figure 2. (A) Fluorescent microscopy images of GFP tagged MSCs at different times upon incubation with the macro-capsule, (B) a comparative graph for corresponding GFP signal intensities calculated by Image J.

For a better understanding of the metabolic activities of living cells in the incubation media, two key biological molecules were analyzed quantitatively. The first one is glucose molecule, which is an essential element, especially for cell proliferation, whereas the other one is lactic acid produced by cells as a metabolic waste molecule during cultivation. Determination of amounts of glucose and lactic acid with respect to incubation time is a well-accepted method for monitoring cellular metabolic activities. The glucose consumption and lactic acid production levels of the cells encapsulated inside the macro-capsule were presented in Figure 3 (A and B, respectively) for 15 days of the incubation period. Samples collected from the incubation solution were analyzed by ADVIA® 1800 Clinical Chemistry System and compared with the results obtained from the 2D cell culture experiment as a control. Alterations in the glucose and lactic acid levels were observed in both 2D cell culture conditions and cells in macrocapsules. Results indicate that the prepared macro-capsules bear a porous architecture allowing the passage of molecules associated with metabolic activity while ensuring the viability of encapsulated living cells inside 3 (A and B, respectively).

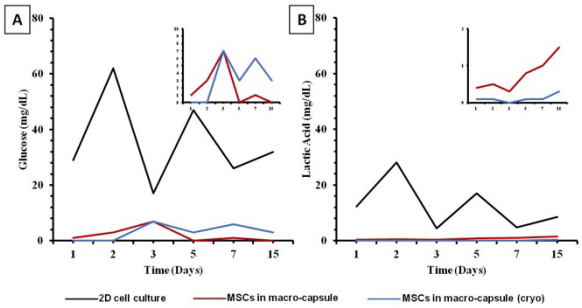

Figure 3. (A) Glucose consumption and (B) lactic acid production profiles of MSCs encapsulated in the macro-capsule.

3.2. Discussion

Mesenchymal stem cell (MSC) therapy has emerged as a highly effective method for the regeneration of damaged tissue and balancing the hormone level inside the body [1–4,19]. Proper isolation of the immune cells might provide ameliorated cell therapy to the patient. Encapsulation of MSCs might be an advantageous strategy for improving the cell survival rate and enhancing cell functioning after transplantation. So, this study aims to construct a polymer-based macro-capsule for the accommodation of cells in an isolated platform once implanted inside the body, which seems to contribute to the increased metabolic activity of the transplanted cell population. A synthetic, yet biodegradable and biocompatible polymer, PCL was utilized for the fabrication of the designed macro-capsule, and it was 3D bio-printed in fiber structure to have permeability ability for the exchange of nutrients reaching the cells inside and removal of waste products out. Obtained results point out that 3D printed PCL-based macro-capsule has enough permeability for the release of HSA, which might enable the proper transfer of albumin proteins inside out for the macro-capsule.

The designed macro-capsule with dimensions of 2x5x10 mm was a small macro-capsule when it's compared to the previously published macro-capsule versions [18]. That is why an injection of a small amount of material with a small-sized syringe needle (insulin syringe, 31 G) creates a big gap in it. The closed form of the macro-capsule might be boomed by gas pressure inside the macro-capsule during the injection of cell solution inside. In addition, the printed form of the macro-capsule contains only a two-layer height which is thin enough for possessing a good permeability property possible. After injection of the cell solution into the macro-capsule, the injection gap was filled with a melted form of PCL to prevent immediate cell release once the macro-capsule was dipped into the incubation solution [20]. The burst release profile is a major indication for showing the leakage formation of the macro-capsule. Leakage

assessment of capsule with HSA protein with a molecular weight of 66.5 kDa (Figure 1C) indicates that protein molecules can pass through the permeable surface of macro-capsule [21].

Although PCL is commonly used in polyester and synthetic polymer for the construction of scaffolds in tissue engineering applications, the major challenge is its cytotoxicity problem emerging from the formation of foreign body response (fibrotic response) when it is in direct contact with the cellular environment [22,23]. Our results show that there is no significant adverse effect of the 'indirect contact' method on the viability of MSCs for 72 hours of incubation time. Direct contact of the macro-capsule to cells has resulted in a slight decrease in a cell. These results suggest that the PCL-based macro-capsule platform might outstand as a convenient carrier of the transplantation of MSCs into the body without decreasing the cell viability significantly after the implantation of the cell-loaded macro-capsule.

The metabolic activity of the encapsulated cells which is intended to be used in protein therapy applications (in the near future) is important to monitor for the secretion of the intended protein at the target site in the body. So, the secretion and release of the metabolic products from inside to outside of the macro-capsule are crucially important for understanding its potential as a biomaterial for the regeneration of the target tissue or balancing of the protein level in the target area. Nutrient and oxygen transition during the cultivation of MSCs has a tremendous effect on cell viability and metabolic activity. In our study, the metabolic activity of encapsulated cells was analyzed by measuring the glucose consumption level and lactic acid production level inside the incubation medium [24]. Alterations in the glucose and lactic acid levels were monitored during the cell incubation process, where the glucose amount in the medium was measured to decrease by almost 10% by time in a newly refreshed media, compared to 2D cell culture conditions. On the other hand, the produced lactic acid was not seen in the medium at the beginning of the incubation process as expected, whereas demonstrated to emerge in the outer media and was detected to be increased in amount by approximately 8% compared to its 2D version at the end of 15 days of the incubation period.

3.3. Conclusions

In this study, polymeric macro-capsule (2x5x10 mm) constructs were fabricated using a polyester-based PCL polymer by an FDM-based 3D bio-printing technology and then analyzed for their different features like their protein release profiles, impact on metabolic activity of encapsulated cells and *in vitro* cytotoxicity levels. A thin layer of porous macro-capsule was investigated for its permeability degree by release studies with different molecules such as trypan blue, and HSA. 98% of the protein was released from the permeable surface of the macrocapsule within 24 hours. Metabolic assessment and viability of cells inside the macro-capsule together with biomaterial toxicity was demonstrated that 3D printed porous macro-capsule platform might be a suitable carrier for patients' stem cell transplantation [7].

Declaration of conflicting interest

The author(s) declared no potential conflicts of interest with respect to the research, authourship, and/or publication of this article.

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