PREPARATION OF PSYLLIUM HUSK POWDER BASED MICROPOROUS COMPOSITE SCAFFOLDS FOR TISSUE ENGINEERING

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Abstract

This study demonstrates the comparison in the method of fabrication and thus evaluates the potential of psyllium husk powder and gelatin-based composite microporous scaffolds for tissue engineering applications. The scaffold is being prepared in three different ratios of 50:50, 75:25 and 100 (w/w of psyllium husk powder and gelatin, respectively) by employing a suitable cross-linking agent, EDC-NHS, followed drying. We have demonstrated the use and outcomes of two different methods of scaffold drying, i.e., vacuum desiccation along with liquid nitrogen dip and lyophilization. It was concluded from the SEM micrographs that the scaffolds dried under vacuum accompanied with liquid nitrogen exposure exhibited less porous architecture when compared to those prepared using a lyophilizer, that resulted in pores in the range of 60-110 μm. Scaffolds fabricated using the former technique lost porosity and sponge-like characteristics of a scaffold. In spite of the above fact, water retaining capacity and stability in the cell culture of such scaffolds is significant, nearly 40-50% of its initial dry weight. Cell culture experiments support the potential of the scaffolds prepared from different methods of fabrication for its cytocompatibility and suitability for cell growth and proliferation for a substantial duration. Erosion in the porous design of the scaffolds was observed after 14 days via SEM micrographs. It was inferred that freeze-drying is a better technique than vacuum desiccation for scaffold preparation. The present investigation has been conducted keeping in mind the importance of drying a scaffold. Scaffold drying is a necessary step to increase its shelf-life, makes it easy to transport and much importantly, controlling the pore size of the scaffold.

Keywords: *Psyllium husk powder, EDC-NHS coupling reaction, microporous scaffolds, hydrogel, freeze--drying, vacuum desiccation*

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Introduction

Tissue engineering is a mixed discipline that mainly focuses on the recreation and regeneration of diseased or damaged tissues [1,2]. Design and development of microporous three-dimensional scaffolds are one of the potential domain of research for tissue engineering and regenerative medicine. Scaffolds form an integral part of tissue engineering as they allow us to mimic native tissue-like environment by complying the structural, biological and mechanical attributes of the extracellular matrix (ECM) present in the native tissues [3]. Very briefly, scaffolds provide an anatomical framework for the cells to reside, the porosity of the scaffold supports metabolite transport efficiently, the strength of the scaffolds is responsible for the mechanical characteristics of the cells, facilitates cell growth and proliferation by providing them enough cues to respond and communicate amongst each other. Therefore, tissue engineering scaffolds are an absolute necessity that provides a conducive environment for cell growth and reproduction with respect to its three-dimensional structure, pore size, strength, cell attachment, degradation rate, etc. [4,5].

Although researchers have access to a plethora of available biomaterials from synthetic such as poly(vinyl alcohol) (PVA), polycaprolactone (PCL), poly (glycolic acid) (PGA), to natural such as cellulose, alginate, gelatin, chitosan, etc., natural biomaterials are particularly being selected for their easy availability, low toxicity, enhanced bioactivity and cytocompatibility [6,7]. With the advancement in tissue engineering the use of plant-derived biopolymers such as alginate, guar gum, aloe gel to name a few, have gained momentum. Easy accessibility, high acceptability and their use in the treatment of human ailments over many decades have made them the material of choice for tissue engineering applications. Plantago ovata or psyllium husk is one of the most widely used and commercially available plant-derived polysaccharides in Indian markets [8]. It has been used in many biomedical applications because of its ease of availability, low cost, non-toxicity, biodegradability, and safety [9]. Gelatin, a versatile and naturally occurring biopolymer helps in the modulation of cell adhesion because of the presence of cell adhering moieties [10-15]. It has been used as an acceptable scaffolding biomaterial for cardiac, hepatic, skin, bone and many such tissue engineering applications [16].1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) has been used as a cross-linking agent as it introduces an amide or an ester bond between the functional moieties of the biopolymers [17-19]. The use of EDC-NHS does not involve any addition of spacer arms between the conjugating molecules as it belongs to a class of zero-length cross-linker [20]. This unique feature of EDC-NHS makes it a potential cross-linker as it does not integrate with the final product and leaves no toxic substance in the fabricated scaffold [21,22].

Scaffold drying is an important step for scaffold preparation. It increases the shelf-life of the material and thus the scaffold, makes it easier to transport, allows a control over the range of pore sizes formed within the scaffold which is a critical feature for cell growth, provides a uniform structure and maintains the integrity of the scaffold and to some extent provides an aesthetic value to the scaffolds [23]. Freeze-drying involves the use of present ice-crystals as a template for pore formation. It applies the principle of sublimation that occurs when the formed ice-crystals go directly to the gaseous state thus omitting the liquid phase. This process allows little control over pore size and interconnectivity between the pores is maintained and prevents the structure from collapsing [24,25].

The main aim of this study is to compare the drying methods of vacuum desiccation and freeze-drying in the fabrication and characterization of psyllium husk powder and gelatin-based three-dimensional microporous scaffolds, cross-linked with EDC-NHS coupling reaction, thus supporting its potential for tissue engineering applications.

Materials and Methods

Psyllium husk powder and gelatin (FIG. 1 a,b) composite scaffolds were prepared by mixing them in the three different ratios, namely 50:50, 75:25 and 100:0 (w/w) respectively, in water. A total of 0.4 g of psyllium was mixed with 14 mL of distilled water to prepare 100% psyllium husk scaffolds. Similarly, the ratios 50:50 and 75:25 were also prepared. The prepared ratios were then cross-linked using 25 mM EDC and 10 mM NHS solution prepared in 95% alcohol solution at an acidic pH, as already explained in detail elsewhere [26] followed by a drying step. Digital photographs of the fabricated scaffolds were captured from a 13-megapixel digital camera. In the present study, one set of scaffolds were dried for two days using a desiccator connected to a vacuum pump accompanied with a liquid nitrogen dip for 10-15 seconds for complete drying. The other set of scaffolds was cross-linked using the same procedure as above except it was dried for two days in a freeze-drier.

Scanning electron microscopy analysis using a Zeiss EVO 18 SEM (Zeiss, Oberkochen, Germany) at 20 kV, was carried out to determine the porous architecture of the scaffolds after respective drying steps. Cell culture studies were performed with L929-RFP (red fluorescent protein) mouse fibroblast cells to identify cell viability, growth, and cell-cell communication within the fabricated scaffold [27]. For freeze-dried scaffolds circular discs of the scaffolds were punched out with 5 mm diameter and similar thickness of the scaffolds was used, i.e. 1-2 mm approximately. Whereas for the scaffolds dried by vacuum desiccation and liquid nitrogen dip had become crispy and hard making it difficult to punch out discs of 5 mm diameter. Therefore, pieces of similar dimensions were cut and used for cell culture experiments. The scaffolds were sterilized by exposing them to ultra-violet radiation for 30 min inside a biosafety cabinet.

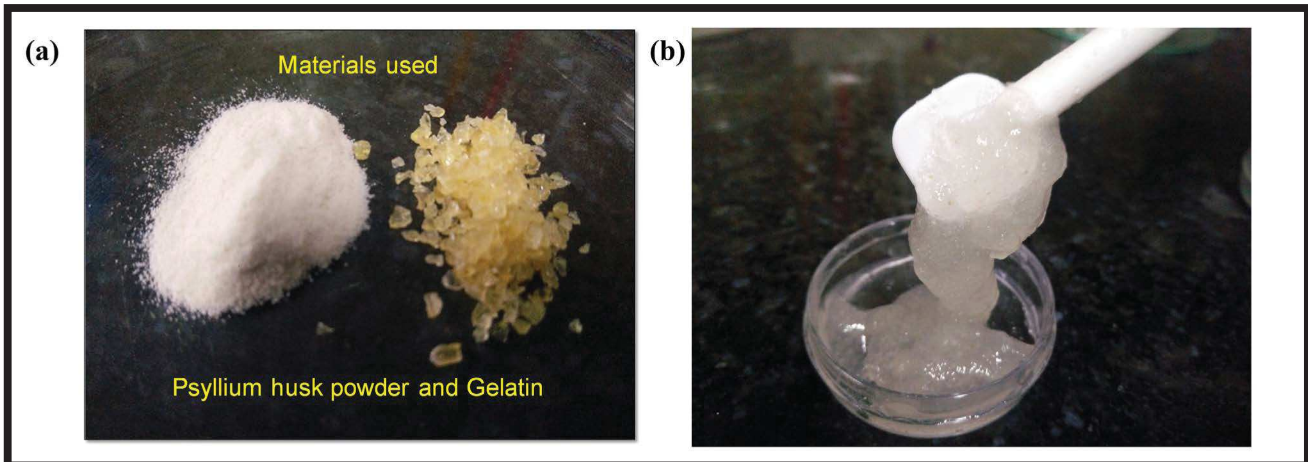

FIG. 1. Representative images of (a) raw materials, i.e. psyllium husk powder and gelatin, used in the fabrication of the scaffold and (b) the viscous mixture formed after mixing the raw materials.

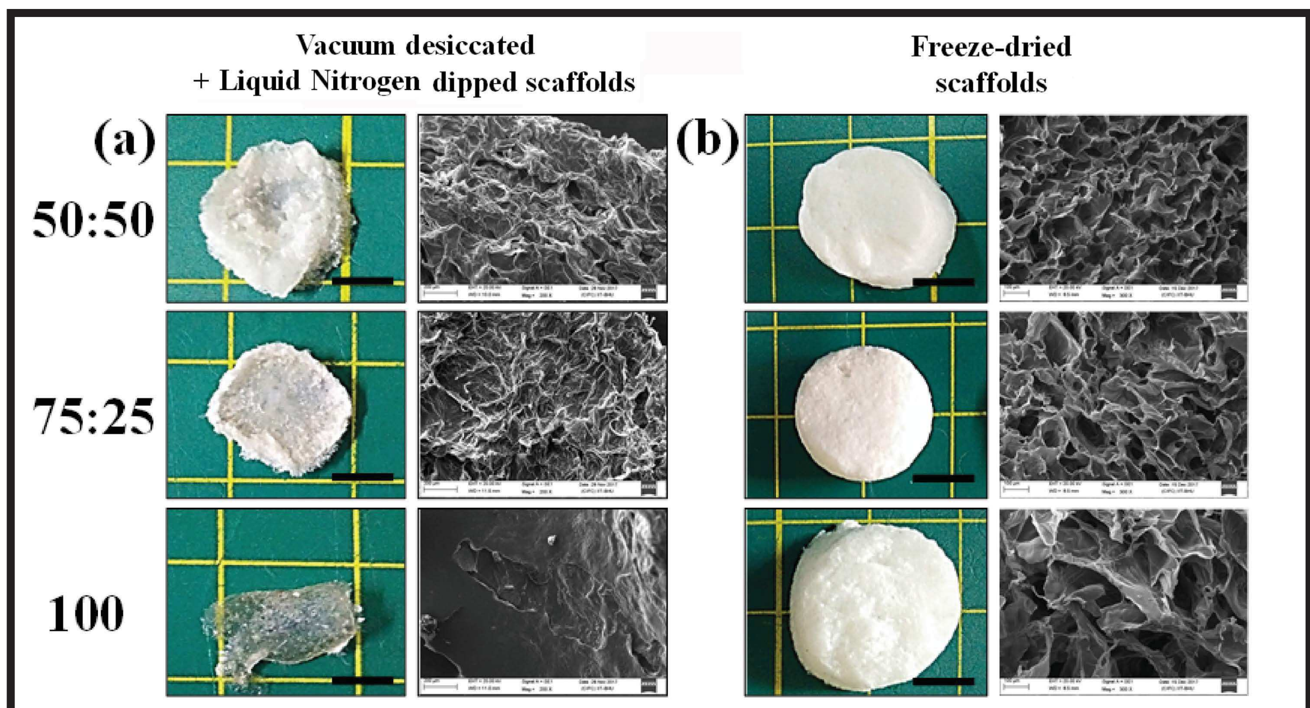

FIG. 2. Digital photographs of fabricated scaffolds by utilizing the two methods for drying the scaffolds and the SEM micrographs that depict porous architecture of the scaffolds. Scale bar: 0.5 cm.

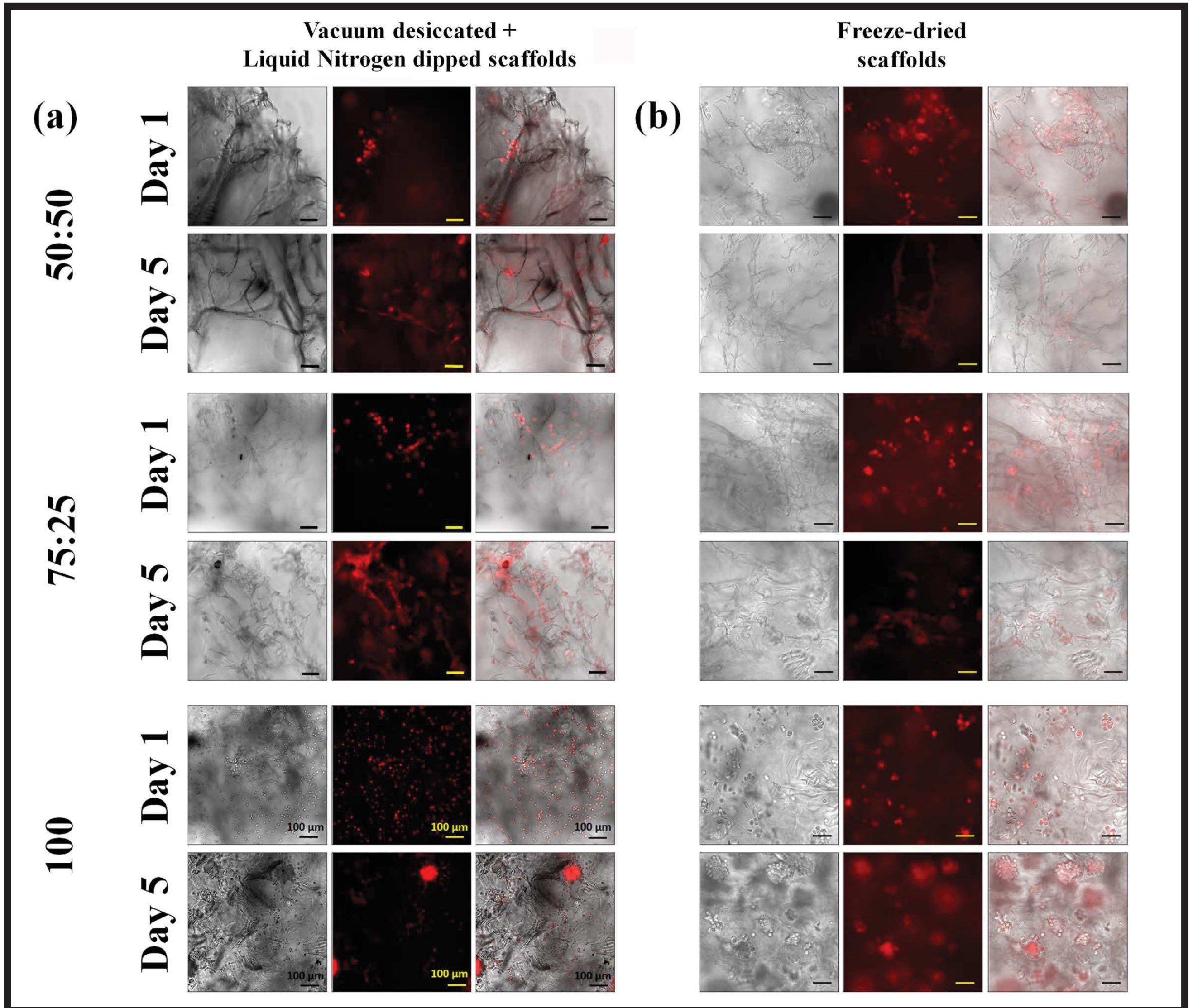

FIG. 3. Panel of images of L929-RFP mouse fibroblast cell culture within the scaffolds prepared from different drying techniques. The panels exhibit brightfield, fluorescent and merged images (from left-right) of the cells within the scaffolds at day 1 and day 5. Scale bar: 50 µm.

Cells were then seeded at a density of 10^5 cells per scaffold and the scaffolds were allowed to soak in the cell suspension solution for about half an hour inside a humidified CO_2 incubator (Galaxy® 170 S, Eppendorf, Germany). The scaffolds were placed in a Petri dish and fed with the optimum volume of cell culture medium that was maintained inside a humidified, 5% CO_2 incubator at 37°C for further experiment. The complete cell culture medium was prepared from Dulbecco's modified Eagle's Medium (DMEM, HiMedia), 10% fetal bovine serum (FBS, HiMedia) and 1% Penicillin/Streptomycin (HiMedia) antibiotic solution. The fluorescent images of L929-RFP cells within the scaffolds were collected at regular intervals using an inverted, DIC, fluorescence microscope (Nikon Ti-U).

Results and Discussions

It is observed from the digital photographs of the fabricated scaffolds that the first set of scaffolds, dried in a vacuum desiccator and exposed to liquid nitrogen, exhibits a contracted or deflated physical appearance (FIG. 2a) that seems to be progressing towards a sheet-like structure with pure psyllium (100) composition whereas the set of scaffolds subjected to freeze-drying protocol maintain the physical aspect and integrity of the scaffold (FIG. 2b). The polyhedral shape of the pores of the freeze-dried scaffolds spanned a range of 60-110 µm. The average pore size for all the three ratios of the scaffold was examined as 77±24 µm. The difference in the physical attributes of the scaffolds prepared from two distinct procedures is due to the fact that vacuum desiccation followed by a dip in liquid nitrogen removed all the moisture from the scaffolds thereby leaving no void space within the scaffolds for it to be considered as microporous as highlighted from the SEM images (FIG. 2a). On the contrary, the freeze-drying procedure exploits water content as a template to create microporous structures within the scaffolds thus maintaining its porous morphology [28]. The basic principle of freeze-drying is the phenomenon of sublimation, that immediately converts solid into a vapour state without passing through a liquid state. The material to be dehydrated is first frozen and then subjected to a high vacuum. The complete procedure of lyophilization takes place at a low temperature and pressure that makes it an excellent method for preserving biological and heat-sensitive samples [29]. It was concluded from the cell culture images in FIG. 2 that scaffolds fabricated from both drying steps show equally good cell growth and proliferation when compared for a period of 5 days. The cellular proliferation was found to be >80% by the 6th day of cell culture in the fabricates scaffolds.

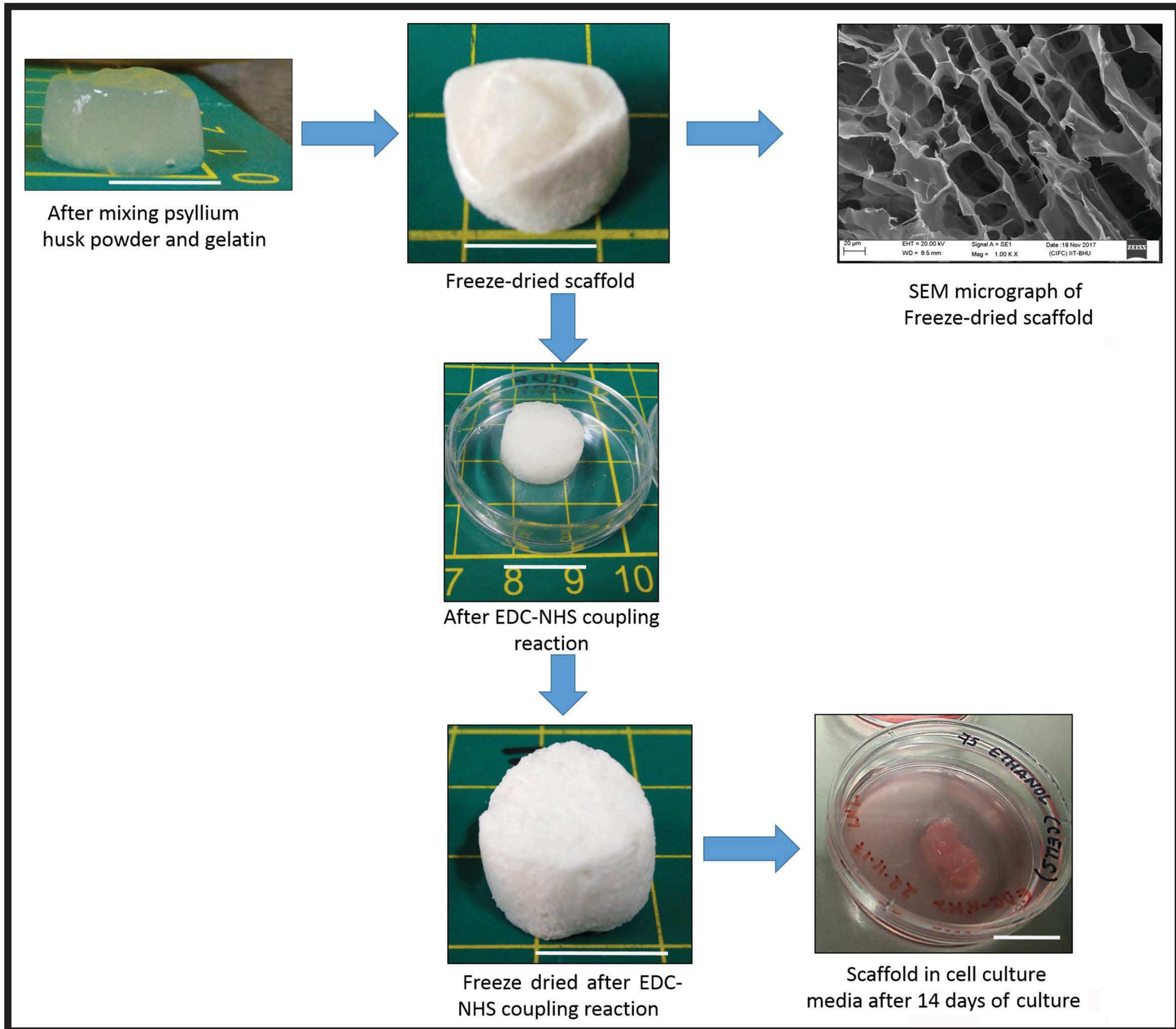

FIG. 4. Representative images of scaffolds at different stages of fabrication process as well as when incubated in culture medium. SEM image shows the internal geometry and microporous nature of the fabricated scaffolds. Scale bar: 1 cm.

The scaffolds dried by vacuum desiccation and liquid nitrogen show commendable swelling in their size when subjected to cell culture media. The reason behind this behaviour may be that while in a vacuum all the air was being sucked out of the scaffold leaving no void space behind which is clearly expressed from the SEM micrographs. Therefore, when a liquid medium was added, it readily absorbed all the solution inside it and distended like a balloon. The swelling capacity or the water imbibing ability of the fabricated scaffolds was investigated as 40-50% of its initial dry weight for scaffolds fabricated from both the techniques. The long-term in vitro degradation of the scaffolds till 14 days in physiological buffer (pH 7.4) at room temperature and at 37°C suggests its resistance towards hydrolytic degradation [30] that makes it a potential candidate for oral and subcutaneous drug delivery systems and tissue engineering applications. It is observed that by day 5 cells made immense network among themselves, a notable cell-cell communication and proliferation is also visible (FIG. 3 a,b). The cells were found to be present through-out the thickness of the scaffold when analysed from Z-stacking images revealing the porous architecture of the scaffolds. The scaffolds were appreciably stable in the cell culture media for a significantly long duration of 3-4 months, although cells were not visible within the scaffolds after 24 days. This indicates that though biodegradable in nature, the degradation of the scaffolds was slow because of its integrity obtained by cross-linking with EDC-NHS coupling reaction. With the given experimental conditions it was inferred that the ratio of 75:25 is most suitable for cell growth, differentiation, and proliferation. The sequence of steps followed in the fabrication process of scaffolds using EDC-NHS coupling reaction as a suitable cross-linker has been represented in FIG. 4.

Conclusions

The outcomes of the conducted study led us to the conclusion that EDC-NHS cross-linked scaffolds fabricated by freeze-drying step exhibit superior porous structure in comparison to those dried in a vacuum desiccator accompanied with liquid nitrogen dip. Although both sets of scaffolds were found suitable for cell growth and communication, structure and integrity of the scaffolds were better maintained in those developed using a freeze-drier, unlike the other set that presented a shrunken appearance. Therefore, in light of the above facts, it was inferred that scaffolds fabricated by the freeze-drying process were more suitable for cell culture and tissue engineering applications.

The fabricated scaffolds can be used in various tissue engineering and regenerative medicine applications such as wound dressing, sustained drug release, three-dimensional microporous scaffold for tissue regeneration and many more, based on the fact that: 1) they have remarkable water imbibing capacity of 40-50% of its initial dry weight, 2) enhanced mechanical strength, i.e. the crispy and brittle nature of the scaffolds obtained from the former technique was replaced by the strength to oppose crushing loads by freeze-drying. The cytocompatibility was determined to be ~80% for L929 fibroblast cells, which also projects its suitability for cell culture. The biodegradable nature of the fabricated scaffold makes it an acceptable platform for the development of bioengineered tissues. Moreover, such an economic type of natural biomaterials are easy to manufacture with minimal toxicity and worldwide acceptance.

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References

[1] V.R. Hokmabad, S. Davaran, A. Ramazani, R. Salehi: Design and fabrication of porous biodegradable scaffolds: a strategy for tissue engineering. Journal of Biomaterials Science, Polymer Edition 28(16) (2017) 1797-1825.
[2] P. Zhao, H. Gu, H. Mi, C. Rao, J. Fu, L. Turng: Fabrication of scaffolds in tissue engineering: A review. Front. Mech. Eng. 13(1) (2018) 107-119.
[3] B.P. Chan, K.W. Leong: Scaffolding in tissue engineering: general approaches and tissue-specific considerations. Eur Spine J 17(4) (2008) 467-479.
[4] F.J. O'Brien: Biomaterials & scaffolds for tissue engineering. Materials Today 14(3) (2011) 88-95.
[5] H. Zhang, L. Zhou, W. Zhang: Control of Scaffold Degradation in Tissue Engineering: A Review. Tissue Engineering Part B: Reviews 20(5) (2014) 492-502.
[6] E. Carletti, A. Motta, C. Migliaresi: Scaffolds for tissue engineering and 3D cell culture. Methods Mol. Biol. 695 (2011) 17-39.
[7] A. Sionkowska: Biopolymeric nanocomposites for potential biomedical applications. Polymer International 65(10) (2016) 1123-1131.
[8] D.M. Mehta, P.K. Shelat, P.B. Parejiya, A.J. Patel, B. Barot: Investigations of Plantago ovata husk powder as a disintegrating agent for development of famotidine tablets. Int J Pharm Sci Nanotechnol 4(2) (2011) 1412-1417.
[9] M.A. Hussain, G. Muhammad, I. Jantan, S.N.A. Bukhari: Psyllium Arabinoxylan: A Versatile Biomaterial for Potential Medicinal and Pharmaceutical Applications. Polymer Reviews 56(1) (2016) 1-30.
[10] A.O. Elzoghby: Gelatin-based nanoparticles as drug and gene delivery systems: Reviewing three decades of research. Journal of Controlled Release 172 (3) (2013) 1075-1091.
[11] N. Davidenko et al.: Evaluation of cell binding to collagen and gelatin: a study of the effect of 2D and 3D architecture and surface chemistry. J Mater Sci Mater Med 27(10) 2016.
[12] S. Gorgieva, T. Vuherer, V. Kokol: Autofluorescence-aided assessment of integration and μ-structuring in chitosan/gelatin bilayer membranes with rapidly mineralized interface in relevance to guided tissue regeneration. Materials Science and Engineering: C 93 (2018) 226-241.
[13] S. Gorgieva, J. Štrancar, V. Kokol: Evaluation of surface/interface-related physicochemical and microstructural properties of gelatin 3D scaffolds, and their influence on fibroblast growth and morphology. Journal of Biomedical Materials Research Part A 102(11) (2014) 3986-3997.
[14] S. Gorgieva, L. Girandon, V. Kokol: Mineralization potential of cellulose-nanofibrils reinforced gelatine scaffolds for promoted calcium deposition by mesenchymal stem cells. Materials Science and Engineering: C 73 (2017) 478-489.
[15] S. Gorgieva, M. Modic, B. Dovgan, M. Kaisersberger-Vincek, V. Kokol: Plasma-Activated Polypropylene Mesh-Gelatin Scaffold Composite as Potential Implant for Bioactive Hernia Treatment. Plasma Processes and Polymers 12(3) (2015) 237-251.
[16] M. Nikkhah, M. Akbari, A. Paul, A. Memic, A. Dolatshahi-Pirouz, A. Khademhosseini: Gelatin-Based Biomaterials For Tissue Engineering And Stem Cell Bioengineering, in Biomaterials from Nature for Advanced Devices and Therapies, Wiley-Blackwell (2016) 37-62.
[17] F. Zhang et al.: Fabrication of gelatin-hyaluronic acid hybrid scaffolds with tunable porous structures for soft tissue engineering. Int. J. Biol. Macromol. 48(3) (2011) 474-481.
[18] S.-N. Park, J.-C. Park, H.O. Kim, M.J. Song, H. Suh: Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide cross-linking. Biomaterials 23(4) (2002) 1205-1212.
[19] T.-W. Wang, M. Spector: Development of hyaluronic acid-based scaffolds for brain tissue engineering. Acta Biomaterialia 5(7) (2009) 2371-2384.
[20] J.M. Lee, H.H.L. Edwards, C.A. Pereira, S.I. Samii: Crosslinking of tissue-derived biomaterials in 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). J Mater Sci: Mater Med 7(9) (1996) 531-541.
[21] K. Jarquín-Yáñez et al.: Structural Effect of Different EDC Crosslinker Concentration in Gelatin- Hyaluronic Acid Scaffolds. Journal of Bioengineering & Biomedical Science 6(2) (2016) 1-6.
[22] B. Kaczmarek, A. Sionkowska, J. Kozlowska, A.M. Osyczka: New composite materials prepared by calcium phosphate precipitation in chitosan/collagen/hyaluronic acid sponge cross-linked by EDC/NHS. International Journal of Biological Macromolecules 107 (2018) 247-253.
[23] N. Zhu, X. Che: Biofabrication of Tissue Scaffolds, in Advances in Biomaterials Science and Biomedical Applications, R. Pignatello, Ed. InTech (2013).
[24] W.-Y. Yeong, C.-K. Chua, K.-F. Leong, M. Chandrasekaran, M.-W. Lee: Comparison of drying methods in the fabrication of collagen scaffold via indirect rapid prototyping. J. Biomed. Mater. Res. Part B Appl. Biomater. 82(1) (2007) 260-266.
[25] D.R. Katti, A. Sharma, K.S. Katti: Chapter 10 - Predictive Methodologies for Design of Bone Tissue Engineering Scaffolds, in Materials for Bone Disorders, S. Bose and A. Bandyopadhyay, Eds. Academic Press (2017) 453-492.
[26] S. Poddar et al.: Fabrication and Cytocompatibility Evaluation of Psyllium Husk (Isabgol)/Gelatin Composite Scaffolds, Appl Biochem Biotechnol (2019). https://doi.org/10.1007/s12010-019-02958-7
[27] N. Varshney et al.: Culturing melanocytes and fibroblasts within three-dimensional macroporous PDMS scaffolds: towards skin dressing material, Cytotechnology 71(1) (2019) 287-303.
[28] G. Nireesha, L. Divya, C. Sowmya, N. Venkateshan, M.N. Babu, V. Lavakumar: Lyophilization/Freeze Drying - An Review. International Journal of Novel Trends in Pharmaceutical Sciences 3(4) (2013).
[29] Basic Cycle Development Techniques For Lyophilized Products [Online]. Available: https://www.pharmaceuticalonline.com/doc/basic-cycle-development-techniques-for-0002.
[30] R.F. Pereira, P.J. Bártolo: Degradation Behavior of Biopolymer-based Membranes for Skin Tissue Regeneration. Procedia Engineering 59 (2013) 285-291.