RESEARCH ARTICLE

Fabrication and characterization of nanofibrous tricuspid valve scaffold based on polyurethane for heart valve tissue engineering

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ABSTRACT

Objective(s): Tissue engineering represents a new approach to solve the current complications of the heart valve replacements by offering viable valve prosthesis with growth and remodeling capability. In this project, electrospinning and dip coating techniques were used to fabricate heart valve constructs from medical grade polyurethane (PU).

Methods: First, a mold of tricuspid valve was dip coated in a PU solution, except for its valvular parts. Then, PU nanofibers were electrospun on the dip coated mold to form the valves. The morphology and diameter of nanofibers were investigated by SEM and contact angle measurements were done to evaluate the wettability of scaffolds. Thereafter, a tensile tester machine was used to assess mechanical properties of nanofibrous scaffolds. Then, the HUVEC cell line was cultured on the surface of scaffolds.

Results: The SEM images showed the proper nanofibrous structure of the prepared scaffolds. Also, the obtained structure demonstrated appropriate tensile properties. Based on direct and indirect MTT, DAPI staining and SEM results, nanofibers were biocompatible and cells attached to the surface of the scaffolds, properly.

Conclusions: This study demonstrated polyurethane-based nanofibrous scaffolds for engineering artificial heart valve. The presented scaffold provides temporary support for cells prior to generation of extracellular matrix (ECM).

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INTRODUCTION

Prevalence of heart valve diseases is increasing in industrial and developing countries. General treatment for end-stage valvular dysfunction is heart valve replacement [1-5]. Three types of valves were known for as replacing heart valve constructs including mechanical, bioprosthetic and polymeric ones. In spite of high stiffness and durability of mechanical heart valves, this type of valves have disadvantages such as poor biocompatibility, thrombosis and thromboembolism, and risk of hemorrhage as a consequence of chronic

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anticoagulation therapy [6, 7]. People who undergo xenografts or allografts bioprosthetic heart valves replacement do not require anticoagulation and therefore do not incur the risks of anticoagulation-related hemorrhage but progressive structural deterioration is the main concern of this procedure [6, 8]. Also, researches on the development of polymeric heart valves are in progress [9]. However, the main disadvantage of these three heart valve replacements is that they cannot grow and remodel. Heart valve tissue engineering is a new approach to overcome the limitations of other methods and is able to promote the new valve growth, remodel and repair regarding the patient [6, 10-13].

The general heart valve tissue engineering involves scaffold fabrication, cell integration and bioreactor conditioning before implantation [14]. To mimic structure of the native heart valve, different types of scaffolds have been tried. Two main kinds of scaffolds have been studied: first, acellular heart valve scaffolds from allogeneic/ xenogeneic sources and second, artificial scaffolds constructed from synthetic and/or natural (biologic) polymers [15]. The considered scaffolds can be further categorized as porous, fibrous, and hydrogel types [16]. Application of the fabricated 3D porous scaffolds is limited due to the lack of ability to mimic the shape and flexibility of native heart valves [16]. Hydrogels show weak mechanical properties and with the addition of cells, their stiffness decrease [17-19]. Fibrous scaffolds are excellent in terms of cell adhesion, migration, proliferation, and differentiation, which are sustantional factors in tissue engineering applications [20-26]. Therefore, fibrous scaffolds would be promising in providing appropriate regenerating heart valves.

Recent studies have shown that electrospun polyurethane (PU) fibers is a proper candidate in soft tissue engineering [27], vascular grafts [28, 29] and scaffolding for promoting neuronal differentiation [30, 31]. Also, promising results were achieved for the application of electrospun polyurethane nanofibers in cardiovascular tissue engineering [32, 33]. In 2009, Xiu-mei MO et al. came up with a novel combination method of electrospinning and rapid prototyping (RP) fused deposition modeling (FDM) that proposed for the fabrication of a tissue engineering heart valve scaffold. [34]. Theirfelder and his colleagues have compared the behavior of seeded cells on synthetic sprayed fibers of polyurethane and biocompatibility of polyurethane scaffold was proven [35]. Recently, elastomeric poly (ester urethane) urea was electrospun onto the rotating conical mandrels. By matching the radius of the conical mandrel to the radius of curvature for the native pulmonary valve, the electrospun constructs exhibited a curvilinear fiber structure similar to the native leaflet [36]. While covering surface of structure with nanofibers improves cell proliferation and spreading, but it needs a supportive backbone to form macrostructure of corresponding tissue. In case of heart valve tissue engineering, lots of studies have synthesized complex and heterogeneous materials to build background of nanofibers.

In this study, nanofibers of polyurethane solution were successfully electrospun and characterized by SEM, contact angle measurements and tensile tests. Then, biocompatibility of nanofibrous scaffolds was evaluated by culturing HUVEC cells and direct and indirect MTT assays. Furthermore, cell-nanofiber adhesion was investigated by SEM. Finally aortic heart valve scaffold was constructed by combination of electrospinning and dip-coating methods so that backbone of scaffold was synthesized by dip coating manner and was covered by nanofibers of polyurethane.

MATERIALS AND METHODS

Electrospinning

In order to fabricate polyurethane nanofibers, the polymeric solution was prepared by dissolving 3 w/w% of a medical grade polyether based PU (TecoflexTM,SG-80A was purchased from Lubrizol USA) in a 50:50 mixture of Chloroform and methanol were obtained from Merck co. (Germany)[37]. After 3 hours of homogenization the solution was transferred to a syringe with a 23-gauge stainless steel cannula. After altering conditions of electrospinning (FNM Ltd., Tehran, Iran), best nanofibers have been achieved when the applied voltage to the electrospinning needle was 20 kV, the distance of the needle from collector was 100 cm and the flow rate of the polymeric solution applied by syringe pump was 1ml/hour. Two kind of mandrel were used to collect nanofibers: (1) rotating cylindrical mandrel was used to form nanofiberous sheets. (2) Rotating heart valve mold, which was designed and manufactured at

the University College London (UCL) was used to synthesis nanofiberous heart valve.

Morphology and fiber diameter characterization

Diameter and morphology of the obtained nanofibrous mats were assessed using scanning electron microscopy (SEM, AIS 2100, Seron, South Korea) after sputtering with gold. Then, 30 fibers in each image were selected randomly and mean diameter of the fibers was measured by Image J software (Sun Microsystems, USA).

Contact angle measurements

Surface hydrophilicity of the nanofibers scaffold and film was investigated by observing water contact angles. The contact angles were evaluated by dispensing a 4µl drop on the samples and analyzing the drop shape. Each measurement was repeated at least three times.

Cell culture and viability test

Human umbilical vein cord cell (HUVEC) was cultured to evaluate responses on the nanofiberous scaffold. HUVEC cells have been chosen to mimic endothelial cells of aortic heart valve (Pasteur Institute of Iran). Cells were plated in tissue-specific flasks 75cm2 and cultured in DMEM-F12 (GIBCO), which was supplemented by 10% fetal bovine serum (FBS) and 1% pen/strep (GIBCO). Also, all cell flasks were incubated at 37˚C and in a 5% CO2 atmosphere. In order to pick up cells, 1 ml of trypsin-EDTA (Sigma-Aldrich) added to flasks and after 10 min incubation, trypsinization ceased by fresh medium.

Cell viability

Based on SEM results, ethanol 70% disrupt surface morphology of nanofibers. So sterilization was done by putting scaffolds were sterilized under UV radiation for 20 min. Scaffolds were next washed several times with PBS containing 5% gentamicin. Then sterilized scaffolds were located in 96-well dishes. After preparing suspension of HUVEC cells in complete media, 10/000 – 12/000 cells were seeded in each well. Incubation time for cells was considered for 24, 48 and 72 hour. We then used direct MTT, which is a calorimetric method to assess the viability of cells on the scaffolds. After 24, 48 and 72 hours incubation time, supernatant were extracted and 100 µl of 5 mg/ml MTT solution in PBS added to each well were incubated in 37˚C and 5% CO2. After 3 hours, it is visible formazan crystal in control well under optical microscope among viable cells. So MTT solution was drawn out and formazan crystals were diluted in 100µl methyl sulfonamide (DMSO). Afterward, this solution was transferred to vacant wells for spectrophotometric analysis at 570 nm in an ELISA reader. The cell viability on nanofibers was evaluated in comparison with control well (TCP) containing just cells. Also, indirect MTT assay was considered for more cytotoxicity analysis. For this purpose, after sterilization of scaffolds, mats of nanofiber were put inside the wells. Then complete media (89% DMEM media + 10% FBS + 1% pen/strep) was added to 96 well plate containing scaffolds and was kept in an incubator for 10 days. Also in another 96 well plate, HUVEC cells were cultured. After 10 days, HUVEC cells were treated by the different proportion of fresh and incubated culture media in which scaffolds were kept for 10 days. After treatment cells incubated for 48 hours and finally MTT protocol was done.

Nucleus staining of the cells

After sterilization as mentioned above, scaffolds were located in 24 well dish and about 70.000 cells were seeded on scaffolds and vacant well as a control and incubated for 48 hours. Then supernatant were extracted and wells were washed three times with PBS. Next, cells were fixed on the scaffolds by paraformaldehyde 4% and 20 minutes. After that, wells were eluted by PBS again and 250µl of DAPI (5µg/ml) was added to each well (a DNA-specific fluorescent probe). Finally, scaffolds and control well, were washed two times and 500µl of PBS appended to wells. Fluorescent microscope (Optika, Italy) was used to visualize nucleus of cells on scaffolds and control wells.

Cell interactions to scaffolds

In order to evaluate attachment, extension and morphology of HUVEC cells on pristine polyurethane nanofibers, after sterilization, approximately 100.000 cells were seeded for 24 hours on the scaffolds in 24 well dish. Then, the supernatant of cells was extracted and eluted 2 times with PBS. Afterward paraformaldehyde 4% were added to wells and washed 2 times with PBS after 20 minutes. Then osmium tetroxide

was shed to scaffolds contained wells and in 4˚C for 90 minutes. The last step to preparing of cell loaded scaffolds for SEM imaging is dehydration by different concentration of ethanol. But as mentioned above, ethanol disrupted the surface of scaffolds. So dehydration process was done by freeze-drier.

Mechanical testing

Nanofiberous and film of polyurethane scaffolds were tested using the uniaxial mechanical Tensometer machine (Model STM-20, SATNAM, Iran) to investigate mechanical properties. Samples were cut into 50 mm by 10 mm rectangular strips. In order to measure the reaction force samples were stretched to failure for 7 mm/min extension rate using a 50 Kgf load cell. Young's modulus, the ultimate tensile strength (UTS; maximum stress at the peak point yield stress (Ys) (stress at which the material begins to deform plastically) and yield strain (Yε) (strain representing yield stress) were measured and considered in comparison study.

Fabrication of heart valve

In this study, aluminium aortic heart valve mold (which was designed and fabricated in UCL) was considered as a template for construction of artificial heart valves. So two methods were chosen: dip coating and electrospinning. First, polyurethane solution 7% w/w was prepared with cholorform as a solvent. The mold was then immersed in polymeric solution, was held 20 seconds and was withdrawn from polymeric solution. Afterward, mold was kept under chemical hood until its solvent was evaporated. At the end of process a visible layer of polymer was coated on the aluminum mold. To increase the diameter of coated polymer on the mold to 1mm thickness, this process were performed at least 10 times. Then the polymeric layer was cut at the leaflet areas. In the next step, coated mold was fixed as a collector on electrospinning apparatus. So all sides of mold should be covered by polyurethane nanofibers. Finally, this artificial heart valve had two textures: polyurethane coat in addition to polyurethane nanofibers in each area and alone polyurethane nanofibers just in leaflets area.

Statistical analysis

The results were computed as the mean±standard error of the mean. The data were analyzed via the Students t-test and repeated measures of analyses of variance (ANOVA) test. A probability of less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

This study has composed of three main parts: synthesis and characterization of electrospun nanofibers of polyurethane, cell response to scaffolds and finally fabrication of heart valve prototype and mechanical properties.

Synthesis and Characterization of polyurethane scaffolds

Electrospun nanofibers of polyurethane were synthesized during electrospinning process in which PU polymer was dissolved in chloroform / methanol (50/50) and underwent to electrospinning process (Fig. 1). Polyether based PU TecoflexTM, SG-80A is a medical grade of polyurethane and its application was studied in tissue engineering. So, the film of aforesaid polymer is biocompatible and biodegradable [38-40]. In order to produce nanofibers of this polymer, the new binary solvent system was established. This chloroform/methanol (50:50) solvent system seems to be beneficial to synthesis electropspun nanofibers of polyurethane because chloroform-methanol solvent system is cheaper than HFIP or HFP and safer than DMF which are regularly used for the preparation of polyurethane solution [42, 41]. According to SEM results, desired fibers of polyurethane indicates porous structure and their diameter can provide the appropriate matrix to grow endothelial cells [43, 44]. The average diameter of the optimized fibers was measured 153±4 nm that was interconnected with high degree of porosity. It should be mentioned that optimum mat of fibers was attained by altering solution concentration and proportion of binary solvents and some electrospinning parameters including applied voltage (kV), nozzle to collector distance (cm) and flow rate of polymeric solution (ml/hour). Optimum fibers were defined as (1) narrowest fibers, (2) the stability of the Taylor cone, (3) spinnable at least for one minute and (4) verifying the minimum numbers of droplets and/ or beads on scanning electron microscopy (SEM) images. Contact angle measurement was tested to distinguish hydrophilicity of film and nanofibers of polyurethane which were 82±2 ° and 125±7°,

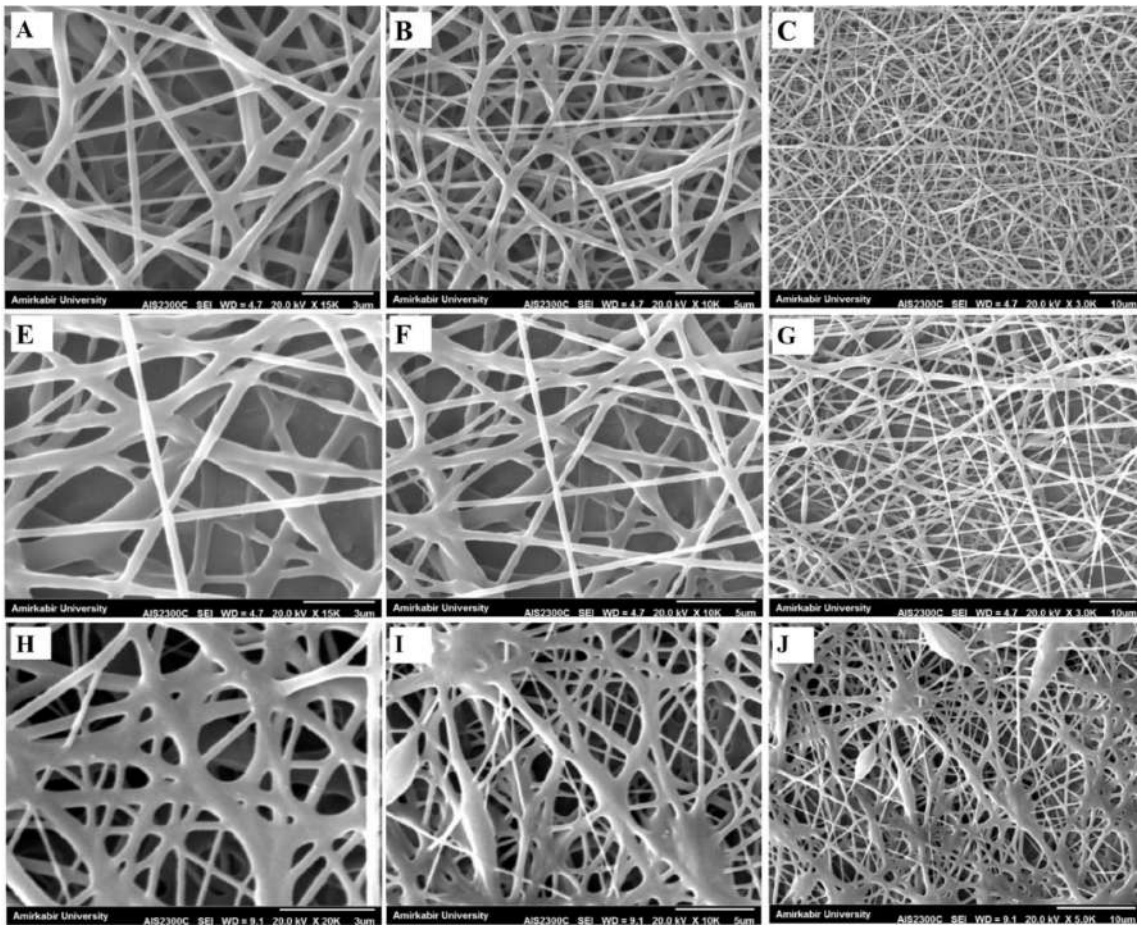

Fig. 1. Electrospun nanofibers of polyurethane which are synthesized by different solvents. (A-C) when polyurethane has dissolved in HFIP. (E-F) when polyurethane dissolved in DMF. (I–H) when polyurethane dissolved in new solvent chloroform/methanol (50:50).

respectively (Fig. 2). Regarding to results, CA of solution cast PU was extended to 82±2 that is near to others reports [45, 46]. In comparison PU film cast, CA of native electrospun nanofibers, which was reported 125±7, showed clearly higher amount. Also, this result was comparable to other studies [47]. Probable reason could be that nanofiberous morphology plays as a role of the resulting interface towards the water drop is a combination of multiple PU and air contact points and the more hydrophobic substrate was attained [48].

Interaction of cells to polyurethane nanofibers
Cells proliferation on the nanofiber

Human umbilical vein cord cell (HUVEC) was chosen to assess biocompatibility of the nanofibers by direct and indirect MTT assays. Direct MTT assay was performed after culturing HUVEC for 24, 48 and 72 hours. In Fig. 3, no significant difference was observed between cells that were grown on the nanofibers and TCP and both mentioned materials showed relative biocompatibility. This data was compatible with indirect MTT results while cells have treated by media, which contained scaffolds for 10 days. So quantitatively evidenced that HUVEC cells had appropriate proliferation on the nanofibers and limited amount of either non-soluble or soluble degradation products substrate was released to media. DAPI nucleus staining was then applied to the cells that were cultured on the nanofibers to assert presence and proliferation of cells on the scaffolds. Explicit population of nucleus was detected on the surface of native nanofibers, which is similar to nucleus of cells that cultured on TCP (Fig. 4). Furthermore our quantitative studies indicated that nanofibers of polyurethane provide non-toxic scaffold for cells growth.

Endothelial cells covering the surface of endothelial

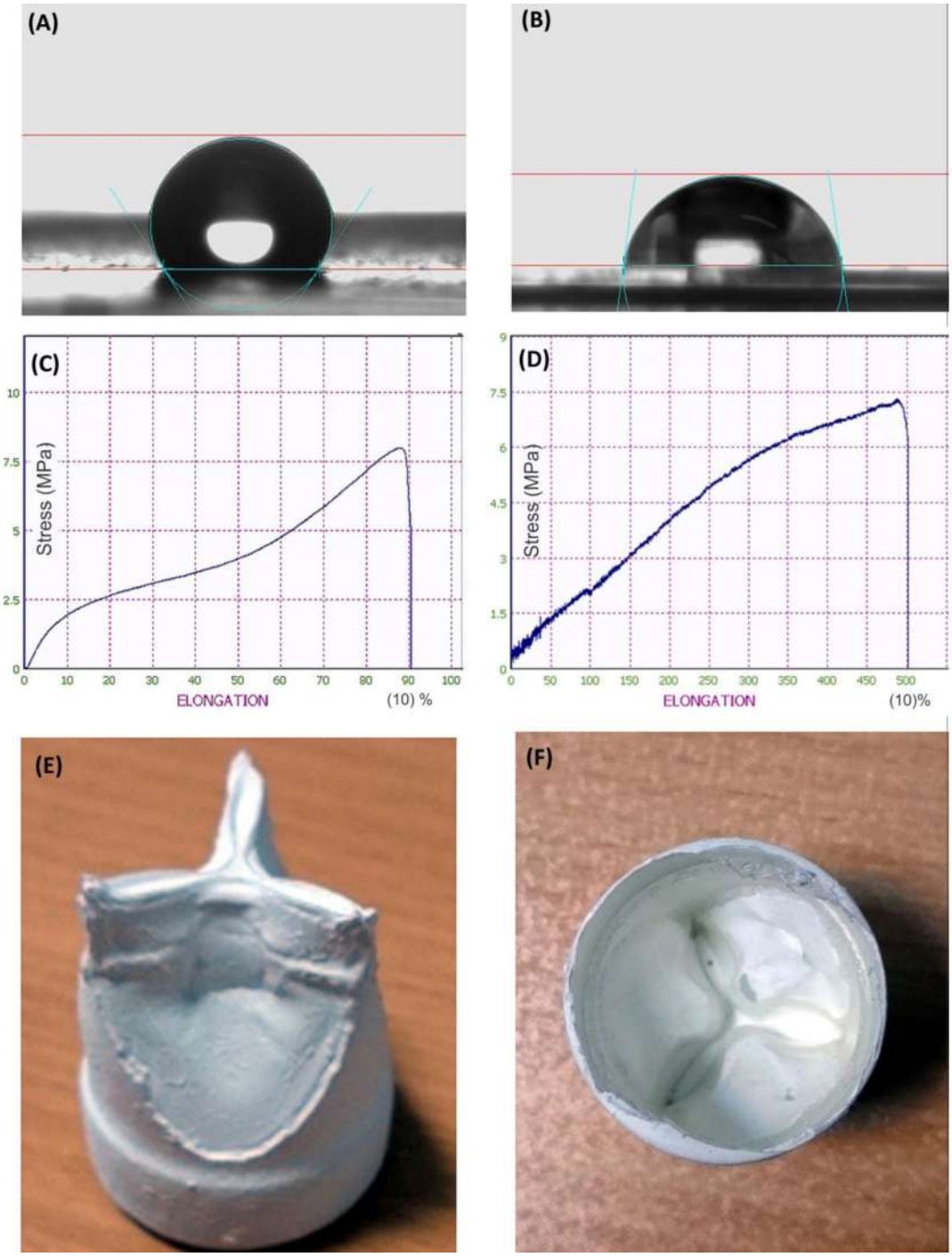

Fig. 2. Contact Angle. (A) Nanofiberous scaffold of polyurethane (B) Film of polyurethane. It is visible that nanofibers were more hydrophobic than film of polyurethane. Tensile mechanical strength of (C) film and (D) fibers of poly urethane. (E) Outside and (F) inside of tissue engineered heart valve which is fabricated by combination of dip coating and electrospinning of polyurethane..

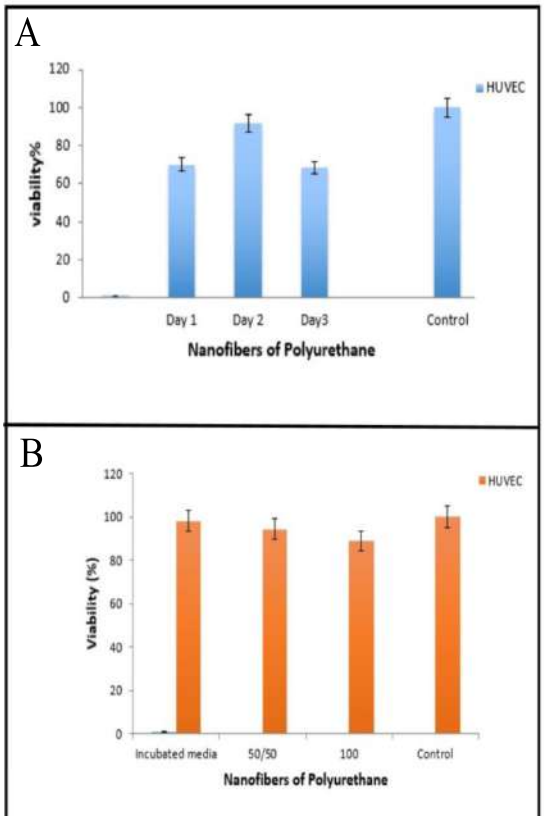

Fig. 3. (A) Direct MTT results obtained after culturing HUVEC cells on nanofibers for 24h, 48h and 72 hours. (B) Indirect MTT results which scaffolds was kept in a complete media for 10 days. Then HUVEC cells which are cultured inside TCP, treated by incubated media at 2 different proportion of fresh and incubated culture media. After 48 hours, HUVEC cells underwent to MTT assay.

cells elsewhere in the circulation, valvular endothelial cells (VEC) maintain a nonthrombogenic blood-tissue interface and regulate immune and inflammatory reactions [49]. layered construct seems proper candidate for valve regeneration [16].Data originated from cellular part of our study showed that nanofibers of polyurethane were not toxic matrix for HUVEC cells. Also, cells were able to expand on the nanotopographical surface of the scaffold. So this nanofiberous scaffold can be a promising matrix for heart valve regeneration. Although further evaluation of scaffold in the case of hemocompaibility, geotoxicicity and phenotype heterogeocity of cells must be performed.

As described previously, natural fibers are main parts of heart valve ECM and both sides of leaflets have covered by fiberous layers (zona fibrosa and zona ventricularis) which are responsible mechanical strength of valves [50]. In our study sinuses and root of fabricated scaffold bilayer component which are film and fibers of polyurethane. The leaflets of heart valve scaffold composed of mats of polyurethane fibers. Then obtained scaffold exactly followed pattern and shape of the mold in all positions. So this manner of scaffold synthesis could be a promising approach to attain macrostructure of the desired scaffold including: shape, concavity, convexity and uniformity.

Cells morphology and distribution on the nanofibers

To study behavior and expansion and HUVEC cells, as a kind of endothelial cells, on the scaffold cells were fixed on the nanofibers as mentioned before. Regarding to Fig. 4, cells demonstrated the typical form of them on the native nanofibers and tend to form a monolayer that almost reaches confluence and appears to be similar to the physiological morphology of endothelial cells, with a cobble-stone like arrangement. This finding is so important because native structure of heart valve has composed of endothelial cells and nanofibers of polyurethane can prepare this possibility for them.

Fabrication of heart valve scaffolds and mechanical properties

Dip Coating

Heart valve mold of aluminium, which was designed and fabricated at department of tissue engineering in university of college of London, was chosen as a template for making heart valve scaffold. Native nanofibers of polyurethane were selected for production of prototype. First, a 10% solution of polyurethane in chloroform was prepared. Next, heart valve mold was embedded in to solution very slowly. After 10 seconds, the mold was brought out and stand up and let it to dry. Minimum bubbles or foam must be made during this level inside polymeric film. Then mold was put under chemical hood until solvent was evaporated. This process was carried out 10 times and consequently 1 mm thickness was reached. At this time, the whole of the valve is covered by polymeric film. So leaflet parts of mold must be removed from polymeric films and. In fact this film plays as a holder to let nanofiberous structure form at the leaflet sides.

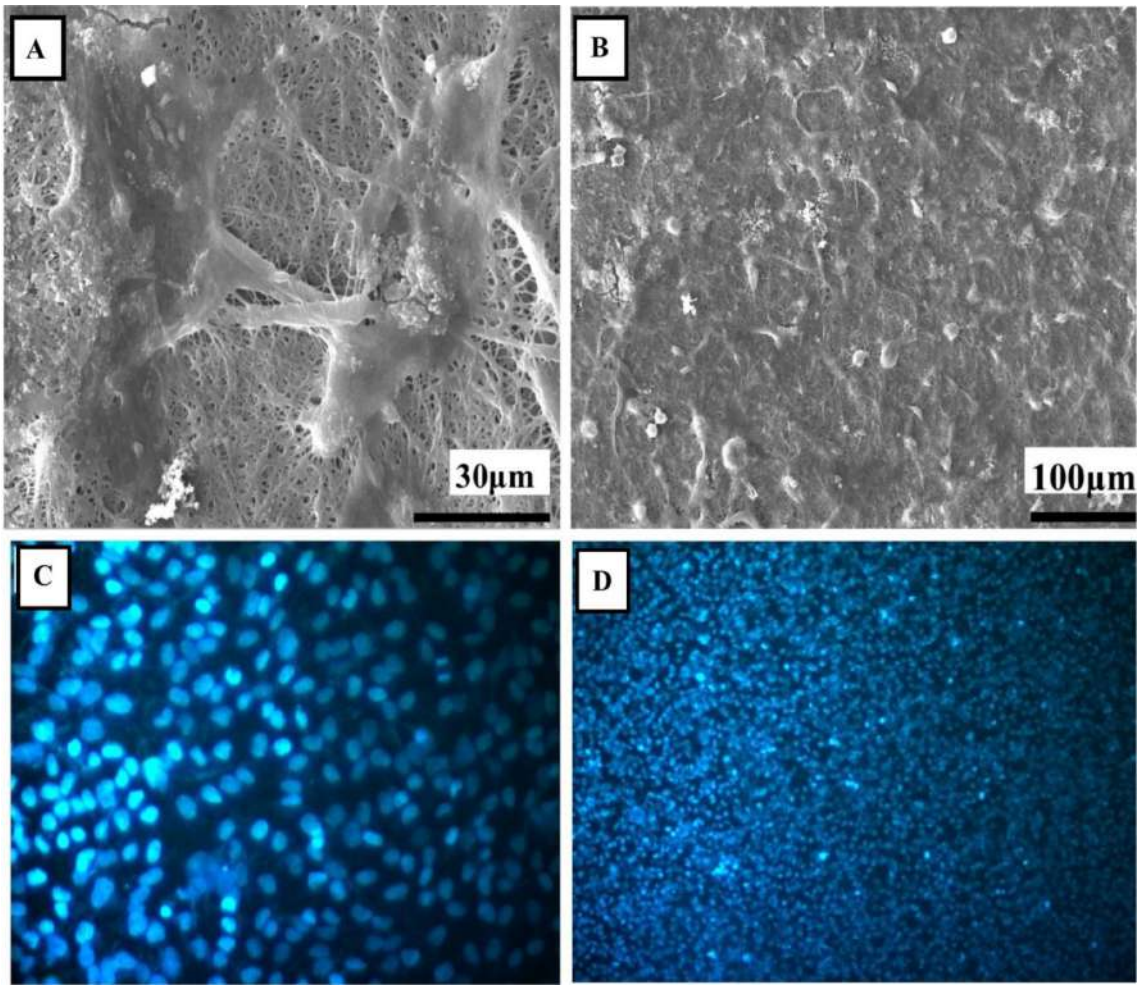

Fig. 4. (A-B) Labeling Nuclear DNA Using DAPI. HUVEC cells which are grown on nanofibers of polyurethane. (C-D) Scanning electron micrographs of HUVEC cells on nanofiberous scaffold.

Electrospinning

At this stage, empty position of leaflets mold must be covered by a nanofiberous layer which its thickness is 200-300 mc. So heart valve mold should be fixed as a collector of electrospinning and rotates with a stable velocity. Like a conical or mandrel types, glow discharge should be happened on the collector. Then the entire heart valve mold was faced with electrospinning of polyurethane nanofibers and other sections between leaflets which had layered of polyurethane film or leaflet parts that were empty were covered by nanofibers of polyurethane. At the end, the scaffold was separated from the mold. Finally a heart valve scaffold was fabricated by combination of dip coating and electrospinning methods (Fig. 2).

Mechanical properties

Tensile test was carried out to distinguish different mechanical behavior of film and fiber of polyurethane. Typical stress-strain curves are shown in Fig. 2 and the testing results are summarized in Table 1. The electrospun polyurethane SG-1080 is drastically thedifferent from bulk one. The bulk type of polyurethane shows regular features of elastomeric materials. Nanofibers of polyurethane are also elastomeric although its diagram is n ot sigmoidal. The curve is steady without any curvature in its slope. Ultimate tensile stress of film and nanofibers of polyurethane was 6.88 MPa and 4.79 MPa, respectively. On the other hand, Young Modulus and the ultimate tensile strain was significantly different. Film of polyurethane was more flexible than nanofibers while Young

Modulus of the fibers was higher.

Uniaxial tensile testing is perhaps the most common tools to measure the mechanical properties of heart valves[51]. As the strain rise, more fibers get in line and the stress in the electrospun scaffold increases. At 300% strain, the stress in the nanofibers of PU increases more rapidly than stress in the bulk. The strain due to organization of nanofibers is illustrated in Fig 2. At equal amounts of strain, stress remains higher in nanofibers until failure at approximately 300% strain. This sharp premature failure is another difference between the two stress–strain curves, as the bulk PU has an ultimate strain to failure of over 700% strain. The primary distinction observed in the stress–strain behavior of the two types of the PU may be clarified by comparing morphology and molecular orientation in each [52].

Thus a large number of studies report the uniaxial tensile properties of both native heart valves and TEHV constructs. However, as noted earlier the static uniaxial tensile tests are too simplistic and do not represent the physiological loading conditions on heart valve leaflets in vivo. For better characterization, dynamic biaxial and flexural properties of leaflets are more relevant[53].

In our study, primary properties of mechanical test are promising. But in order to further evaluation of scaffold dynamic biaxial and flexural experiment should be performed. Heart valve is being faced lots of turbulence during cardiac cycle. So, investigation of mechanical features of heart valve scaffold, when cell loading increases and while the scaffold is degrading in a suitable bioreactor, is necessary.

CONCLUSIONS

In the present study, a synthetic heart valve was successfully fabricated utilizing electrospinning and dip coating techniques. The results demonstrated that this special nanofibrous valvular structure would sufficiently support cell attachment and viability. Also, polyurethane-based structure would render the obtained structure with appropriate mechanical properties. This valvular construct would be promising in the future heart valve reconstructions.

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CONFLICTS OF INTEREST

The authors certify that there is no conflict of interest.

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