Biocompatible Scaffolds from Chitosan/Cellulose Acetate & Blends therefrom

Mona M. Naim, Mahmoud M. Elewa, Mervat A. Abdelkawi, Nouran A. Milad*

Abstract— Tissue engineering is a very important field in biomedical applications such as in skin regeneration, nerves, liver, cartilage, heart valves, etc., of which the essential requirements are to prepare suitable scaffolds that are biocompatible, biodegradable and of uniform pore-size. Natural biocompatible polymers such as chitosan (CS) have been often explored in the preparation of scaffolds, that are later seeded with cells, in bioreactors, for replacing burnt or damaged skin. In addition, regenerated cellulose, such as cuprophane, has been the membrane of choice for blood purification by the artificial kidney, for decades. Accordingly, in this work scaffolds were fabricated from either CS or cellulose acetate (CA), or blends therefrom, by a simple novel laboratory technique, in which the polymer/polymer blend is dissolved in a suitable solvent/solvent mixture, to form solutions which are cast by a special casting assembly into membranes, such that pores and scaffolds are created, by forcing humid air to flow along the as-cast membrane. CS was prepared from shrimp shells through deacetylation with concentrated sodium hydroxide (NaOH) solution. Variables investigated were: type of polymer, type of solvent, initial polymer solution concentration, concentration of deacetylation solution, and time of exposure to humid air flow of as-cast membrane. Scanning Electron Microscopy (SEM) examinations were conducted to determine membrane surface and cross-section morphologies, and it was found that CS gave a suitable surface and that the blend membrane gave a better membrane as regards the number of pores, while CA gave promising scaffolds. Moreover, the type of solvent affected the membrane morphology, and deacetylation with 50% NaOH solution gave a more uniform CS membrane than lower concentration. Moreover, it was shown that a 30 min exposure time gave a membrane with more scaffolds/pores >20 min >10 min. The scaffolds suggest that they might be suitable for formation of replacement skin tissue, after cell seeding, in future work, which is currently proceeding in our lab.

Keywords— Biocompatibility, Biodegradability, Bioreactors, Cellulose acetate, Chitosan, Membranes, Scaffolds, Tissue engineering.

1 Introduction

TISSUE engineering consists of a multidisciplinary science, including fundamental principles from materials engineering and molecular biology in efforts to develop biological substitutes to replace damaged or defective tissues and organs. In the most general sense, tissue engineering seeks to fabricate living replacement parts for transplantation and reconstructive surgery for the body [1]. Both materials and fabrication technologies, are critically important for tissue engineering in designing temporary, artificial extracellular materials matrix ECM (scaffolds), which support three-dimensional tissue formation [2]. There are three approaches in tissue engineering: the use of isolated cells or cell substitutes to replace those cells that supply the needed function; the delivery of tissue inducing substances, such as growth and differentiation factors, to targeted location, and growing cells in three dimensional scaffolds [2].

Scaffolds are three-dimensional and highly porous structures with the majority of pores connected to each other. They are mainly used as templates to direct the growth of tissue in the body, or as delivery vehicles for transplanted cells or drugs in an attempt to regenerate structural, and eventually load-bearing tissue. Therefore, these scaffolds may be implanted into a tissue defect without any cells or bioactive compounds previously incorporated, and the tissue regeneration depends on the in-growth of the surrounding tissue only. Alternatively, the scaffolds may be loaded with cells or compounds, before their implantation, to improve the rate of tissue in growth, vascularization, and cell differentiation [3].

A number of fabrication technologies have been applied to process biodegradable and bioresorbable materials into 3-D polymeric scaffolds of high porosity and surface area [4]. The conventional techniques for scaffold fabrication include fiber bonding [5],[6], solvent-casting particulate-leaching [3],[4],[5],[7], phase separation [4],[5],[8], membrane lamination [4], gas foaming [4],[5],[9], freezing [4],[5],[10], solution casting [5],[11],[12], using a porogen [5], emulsion freeze drying [4],[5],[13], and melt molding [4],[5],[14]. However, solvent casting in combination with particle leaching, works only for thin membranes or 3-D specimens with very thin wall sections [4].

The present generation of tissue engineering research is based on the seeding of cells onto porous biodegradable polymer matrices. A primary factor is the availability of good biomaterials to serve as the temporary matrix. These biomaterials must be capable of being prepared in porous forms to offer

- *Mona M. Naim is professor in Chemical Engineering Department, Faculty of Engineering, Alexandria University, Alexandria, Egypt, PH-01223327112, E-mail: monanaim66@gmail.com*
- *Mahmoud M. Elewa is Director of Applied Research Center, Arab Academy for Science, Technology and Maritime Transport, Alexandria, Egypt, PH-01222822442, E-mail: mahmoud.elewa@gmail.com*
- *Mervat A. Abdelkawi is associate professor in Institute of Graduate Studies and Research, Alexandria, Egypt, PH-01224879931, E-mail: mervat_kawi@yahoo.com*
- *Nouran A. Milad is currently pursuing Doctorate degree program in Chemical Engineering Department, Faculty of Engineering, Alexandria University, Alexandria, Egypt, PH-01007566535, E-mail: nouran_ahmed@yahoo.com*

a channel for the migration of host cells into the matrix permitting growth into complete tissue analogues and be biodegradable into non-toxic products once they have served their function *in vivo* [15].

CS has been investigated for a great number of biomedical applications [16] including tissue engineering scaffolds. It is the most potent candidate as a scaffold for skin substitutes due to its physico-chemical and biological properties [1]. Considerable attention has been given to CS-based materials and their applications in the field of tissue engineering as it is biocompatible, biodegradable, can be molded into porous structures, and has intrinsic antibacterial activity [1],[2]. Individuals who have suffered extensive losses of skin, commonly on fires, are actually ill and in danger of succumbing either to massive infection or to severe fluid loss.

Malette et al [17] studied the effect of treatment with CS and saline solution on healing and fibroplasias of wounds made by scalpel insertions in skin in the abdominal surface of dogs. Yannas et al [15] proposed a design for artificial skin, applicable to long-term chronic use, focusing on a non-antigenic membrane, which performs as a biodegradable template for synthesis of neodermal tissue. It appears that CS, having structural characteristics similar to glycosamino glycans, could be considered for developing such substratum for skin replacement.

Recently, Mizuno et al [18] also reported that CS was a good wound healing material and incorporation of that to basic fibroblast growth factor (bFGF) accelerated the rate of healing. Howling et al [19] demonstrated that highly deacetylated CS are more biologically active than chitin and less deacetylated CS. Further studies emphasized on the combination of CS with other materials which have a potential way of achieving rapid wound healing. Yan et al [20] prepared biodegradable CS-alginate polyelectrolyte complex (PEC) membranes, which showed greater stability to pH changes and hence more effective as controlled-release membranes than either CS or alginate itself [21]. The PEC membranes were found to promote accelerated healing of incisional wounds in a rat model [1].

Ma et al [22] fabricated porous CS/collagen scaffolds with improved biostability and good biocompatibility by their cross-linking with glutaraldehyde (GA) and freeze-drying. They also reported that the potential cytotoxicity of GA might be decreased through the presence of CS, i.e. CS can increase the cross-linking efficiency of GA in the collagen-based scaffolds owing to the large number of amino groups in its molecular chain.

Malafaya et al [23] managed to fabricate CS scaffolds based on a particle aggregation technique that fulfilled three main key requirements for tissue engineering scaffolding: morphological adequacy, mechanical stability and *in vivo* functional biocompatibility. The developed scaffolds had an adequate pore size range and an interconnected porous structure.

To combine the individual advantages of synthetic and natural polymers, poly (L-lactic acid) (PLLA)-CS hybrid scaffolds were fabricated by Jiao et al [24]. PLLA sponges were prepared by particulate-leaching and then PLLA-CS hybrid scaffolds were obtained by dipping the PLLA sponges in CS solution and subsequently freeze-drying. Physicochemical properties of the scaffolds were characterized by SEM, water uptake tests and mechanical strength measurement. Their study suggested that the hybrid scaffolds obtained good mechanical strength from PLLA and excellent cell compatibility from CS.

Duarte et al [25] also managed to prepare novel 3D porous scaffolds of blends of CS and PLLA, using supercritical fluid technology to assist phase-inversion. Samples with different polymer ratios and different polymer solution concentrations were subjected to supercritical assisted phase-inversion experiments. It was concluded that porosity is greatly influenced by the concentration of polymer in solution and on the ratio of CS:PLA. The presence of a very high concentration of PLLA in the blend produces structures exhibiting bigger and more heterogeneous pores. The method consisted of a clean and environmentally friendly technology constituting a new processing technology for the preparation of scaffolds for tissue engineering using these materials.

Wan et al [16] fabricated novel porous scaffolds which showed well distributed and interconnected porous structures with controllable porosities varying from around 50 to 85%, using biodegradable polylactide/CS blends, by a combinational technique involving solvent-extracting, liquid-solid separation, and freeze-drying paths. The processing parameters were optimized in order to produce desired porous scaffolds. Increasing the weight ratios of CS resulted in a scaffold with higher porosity and larger pore size.

A novel electrochemical method of preparing CS scaffolds with three different molecular weights in various acid (acetic- and formic- acid) solutions was reported by Twu et al [26]. CS samples prepared by electrolysis in acid solutions exhibited a rod-like geometry and a porous morphology with detectable deacetylation and degradation. The recovery rate of high molecular weight CS was highest among three polymers in all types of solvents. The authors proved that CS scaffolds lost some degree of crystallinity and showed a porous structure (pore size 0.5 – 2 mm).

Mao et al [27] prepared CS-gelatin hybrid polymer network scaffolds via the freeze-drying technique by using the ice microparticle as a porogen, by which monolayer and bilayer scaffolds

were obtained by using different pre-freezing methods.

Jayakuma et al [28] focussed on the preparation, characterization, bioactivity and biodegradation of chitin-chitosan nano-ZrO_2 composite scaffolds in detail. The biomedical grade of ZrO_2 is considered to be one of the most used materials after titanium. Zirconium is widely used to build prosthetic devices due to its good mechanical strength and biocompatibility. At tissue level, Zirconia was found to be as biocompatible as titanium. All the results suggested that the developed nano-composite scaffolds possessed the pre-requisites for tissue engineering scaffolds and could be used for various tissue engineering applications.

A facile approach to construct three-dimensional oriented CS scaffolds with an in situ precipitation method was adopted by Li et al [29]. The CS gel with multilayer structure was first prepared with in situ precipitation, and then lyophilization was applied to obtain porous scaffolds. SEM images indicated that the porous scaffolds had spoke-like frame work in cross-section and multilayer structure in vertical section.

Bio-inspired bi-layered physical hydrogels comprising CS and water only, were processed by Boucard et al [30] and applied to the treatment of full-thickness burn injuries. A first layer consisted of a rigid protective gel ensured good mechanical properties and gas exchanges, while a second soft and flexible layer allowed the material to follow the geometry of the wound and ensured a good superficial contact. For comparison, highly viscous solutions of CS were also considered. Their preliminary study essentially demonstrated that CS based materials were totally accepted by host organisms. It also showed that CS gels and viscous solutions allow the dermis and dermal- epidermal junction reconstruction and the epithealization on the full thickness skin defect. Its porosity was sufficiently low to preclude any physical transfer of living cells and ECM throughout its structure.

In a study by Kim et al [31] bioactive molecule immobilized CS scaffolds with controlled pore architectures for enhanced viability of human mesenchymal stem cells were developed. The molecular weight of CS was decreased by ultrasonication of CS solution, which was effective in the formation of porous CS scaffolds, resulting in an increase of interconnecting micro-pores (~ 10 μm) between macro-pores.

Sawaguchi et al [32] assessed the effect of various cyclic mechanical stresses on cell proliferation and extra cellular matrix production in a 3D scaffold made from CS and hyaluronan for ligament and tendon tissue engineering.

Novel 3D scaffolds composed of polyethylene oxide (PEO) and CS was fabricated by Kuo and Hsu [33]. Porosity, moisture content, mechanical extensibility, and bio-degradation rate of the PEO/CS scaffolds increased directly with the PEO content. However, the Young's modulus and the compression modulus decreased as the PEO content increased.

In the present work, scaffolds were fabricated from CS, CA, or blends therefrom, by either phase-inversion or a simple novel technique which involves blowing humid air along the as-cast membrane for a certain time interval prior to phase inversion. The effect of type of polymer/polymer blend, type of solvent or solvent mixture, time of exposure to humid air flow, and method of membrane fabrication, on the introduction of scaffolds/pores into the membrane, determined by SEM examination which will clarify the surface topology and cross-section morphology of the membrane, will be investigated. It is noteworthy that blending CS with CA has never been done before, for the sake of preparation of biocompatible/biodegradable scaffolds, to the best of the authors' knowledge. CA was used for its excellent film-forming properties and to provide mechanical strength to the scaffolds, while CS is the most potent candidate as a scaffold for skin substitutes [1].

2 Experimental Work

2.1 Preparation of chitosan from shrimp shells

2.1.1 Materials used

Shrimp shells were obtained from the local fish market. NaOH flakes (product of Femico, Egypt) were used in the preparation of caustic soda for deacetylating chitin in the shells to CS.

2.1.2 Methods

The shrimp shells were washed well with soap and water after removing any adhering flesh, then washed thoroughly with distilled water and left to air dry on white kitchen paper towels for two days. A concentrated NaOH solution (40 or 50% concentration) was prepared and poured over the CS flakes, then left to boil gently at 70 °C for two hours at constant volume by covering with a watch glass, with occasional stirring using a glass rod, then the liquor was decanted and the CS flakes filtered and washed with distilled water successively by repetitive soaking and decantation till the wash water became neutral (pH=7). Finally, the flakes were left to air dry as before.

2.2 Fabrication of membranes:

2.2.1 Materials used

CS is prepared in our lab (see section 2.1). CA flakes (product of Panreac, Egypt) were used for the preparation of regenerated CA (CDA) membranes. Acetic acid (AA), Chloroform (CF), Acetone (A), Dimethylformamide (DMF) and Formic acid (FA) (products of Adwic, Egypt) were used for dissolving the polymers. Also, Dioxane (D) (product of El-Gomhoria Company) was used as a solvent.

2.2.2 Methods

Two techniques were applied to determine which is the preferred method for introducing pores and/or scaffolds in the membrane matrix, and these are explained in the following paragraphs:

2.2.2.1 Phase inversion: in which the membrane is prepared by casting the solution using a casting assembly, after which the glass sheet with the membrane on it, is immersed in a pan containing lukewarm water for 1 hour to effect coagulation.

2.2.2.2 Exposure to humid air flow: in which the as-cast membrane is immediately exposed to humid air flow using an air blower over a deep pan of boiling water for the desired time in which the plate is rotated clock-wise around its four edges, to assure that all the membrane surface is affected equally with the humid air, after which the plate is immersed in a pan containing lukewarm water for a maximum of 60 minutes, to effect complete coagulation (see Fig.1).

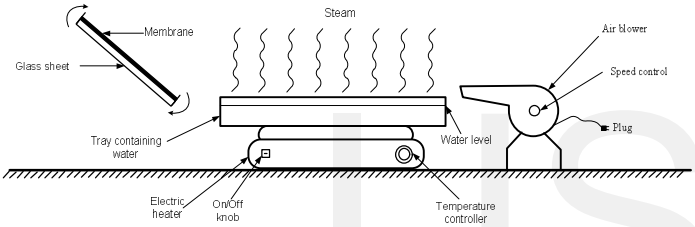
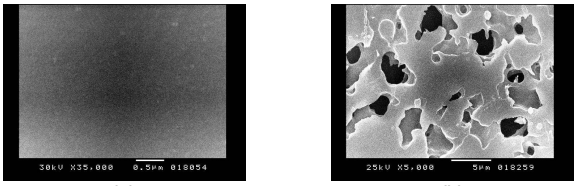


Fig. 1 Schematic diagram of set-up for membrane exposure to humid air flow

3 Results and Discussion

3.1 Effect of membrane fabrication technique

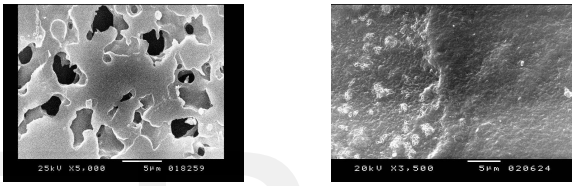
The effect of exposure to humid air flow could be realized on comparing Figs. (2a and b) to each other, from which it is observed that exposure to humid air flow (Fig. 2b) even though for 10 minutes caused the surface to be filled with micro-pores and some mini-pores going deep into the membrane matrix. On the other hand, the other membrane surface (Fig. 2a) which did not undergo exposure to humid air flow contained no pores whatsoever, in the spite that the magnification is much larger than the magnification in the previous figure (35,000 compared to 5,000). The reason for the formation of pores in the first case seems to be due to solvent evaporation from the surface, which on prolonged treatment leads to the formation of scaffolds inside the membrane matrix, as shown in Fig. (2b). Accordingly, it could be stated that exposure to humid air flow induces the formation of pores/scaffolds in the membrane, which matches the observations of Maruyama et al [34] that pore diameter increases with humidity (approximate pore size of 3.5, 4.5, and 5.2 mm for 70, 80 and 90% relative humidity, respectively).



(a) (b)

Fig. 2 SEM micrographs of CS membranes surfaces (6% CS in 2% AA), fabrication technique: (a) evaporation to dryness, (b) exposure to humid air flow followed by evaporation to dryness

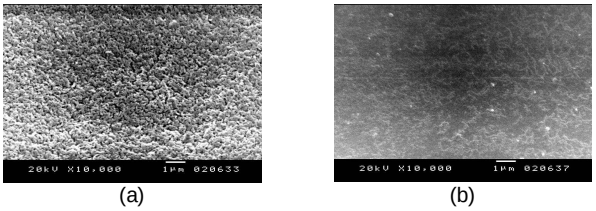
The same effect on the surface morphology of CS membrane is presented in Figs. (3a and b) in which the first has been exposed to humid air flow for 10 minutes while the second was not. It is clear that exposure to humid air flow induces the formation of pores/scaffolds in the membrane as shown in Fig. (3a). On the other hand, Fig. (3b) clarifies the absence of pores or scaffolds which emphasizes the importance of the present treatment on the surface morphology, however nano-particles are visible but which are worthless in such a case.



(a) (b)

Fig. 3 SEM micrographs of CS membranes surfaces (6% CS in 2% AA), fabrication technique: (a) exposure to humid air flow followed by evaporation to dryness, (b) evaporation to dryness

The effect of exposure to humid air flow of an as-cast CDA membrane is demonstrated in the micrograph of Fig. (4a) which reveals the presence of nano-particles on the surface with nano-pores embedded between them, as well as nano-rods which must be due to the long chains of the CDA molecules. On the other hand, Fig. (4b) which depicts the micrograph of a similar membrane which has not been exposed to humid air flow, shows that the surface does not contain any visible pores, yet some nano-rods and nano-particles are shown, but altogether the surface is not homogeneous as that shown in Fig. (4a). Accordingly, exposure to humid air flow is favoured since in addition to the homogeneity of the membrane surface, the presence of nano-pores and nano-rods cause the cells to attach to the surface and the consequent proliferation inside the membrane matrix [35].

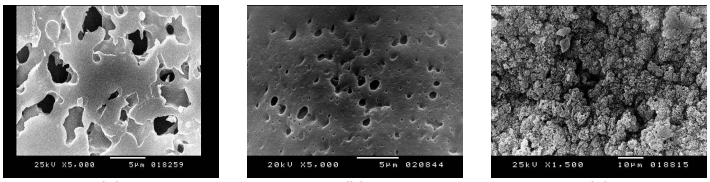


(a) (b)

Fig. 4 SEM micrographs of CDA membranes surfaces (CDA 24.88 g in DMF 120 mL + CF 120 mL), fabrication technique: (a) exposure to humid air flow followed by phase inversion, (b) phase inversion

3.2 Effect of type of polymer/polymers blend

The effect of type of polymer/polymers blend is illustrated on comparing Figs. (5a, b, and c) to each other, and in which CS, CDA, and a blend of CS plus CDA were used as membrane, respectively. The magnifications were 5,000, 5,000 and 1,500 times in respective order. However, the solvents were completely different from each other. Nevertheless, it was observed that the CS membrane as well as the CDA membrane shown in Figs. (5a and b) both contained micro- and a few mini- pores, however the CS membrane contained scaffolds on the surface whereas the CDA membrane did not contain any scaffolds. This observation shows that the type of polymer has an effect on the morphology of the membrane, even though both were exposed to humid air flow. On the other hand, Fig. (5c) indicates a membrane made from a blend of CDA plus CS, from which it is observed that clusters of micro- and mini-particles are present on the surface with few pores and in which no scaffolds are apparent, although the concentration is low. Accordingly, it could be stated that different polymers/polymer blends give different surface morphologies and could affect the formation of scaffolds. However, it should be mentioned that CS and deacetylated CDA are both biocompatible and biodegradable [36],[37],[38],[39],[40],[41],[42],[43],[44]. As emphasized before according to Bartis and Pongrácz [45], further advantages of natural biomaterials in biomedical tissue engineering, than synthetic, are that they already have binding sites for cells and adhesion molecules so the biocompatibility is not a major issue. Lim and Halim [41] also figured out that the cationic amino groups of chitosan bind to anionic groups in some microorganisms, such as fungi, algae and bacteria, resulting in growth inhibition. Therefore, the development of wound dressings from traditional passive materials was replaced by active dressings that create and maintain a moist, healing environment [41],[46].

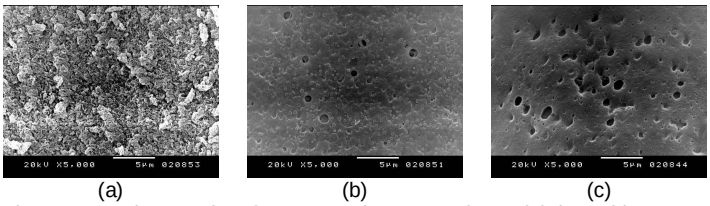


(a) (b) (c)

Fig. 5 SEM micrographs of CS, CDA, and CS/CDA membranes surfaces fabricated by exposure to humid air flow followed by phase inversion, solution composition: (a) 6% CS in 2% AA, (b) CDA 10 g in (CF 88 mL + D 50 mL), (c) (CS:CDA = 1:1 w/w) in (FA:DMF = 1:1 b.v.)

3.3 Effect of type of solvent

An investigation on the type of solvent in case of a CDA membrane is noticed on examining Figs. (6a, b and c) which present the surface micrographs of the membranes which have been exposed to humid air flow for 60 minutes, and in which DMF, D and CF plus D were used as solvents, respectively, at rather different concentrations, being more concentrated in case of DMF. It is observed that the surface, in case of DMF as a solvent, is rough and contains many nano-particles some of which are agglomerated to form clusters of micro-particles; however, the presence of nano-voids is not as clear as in the case of the D solvent. On the other hand, micro- and nano-pores are present in case of D as solvent, but which are covered with plenty of microorganisms during steeping in distilled water till SEM examination, causing the pores to be obscured. However, it is worth mentioning that D had proven earlier to be a promising solvent in forming pores/scaffolds, but since the membrane in Fig. (6b) was formed from a more concentrated casting solution, which had also been proven before to be undesirable, therefore the two last effects nullified each other leading to the present situation. In addition, some inner scaffolds are apparent within the larger pores.



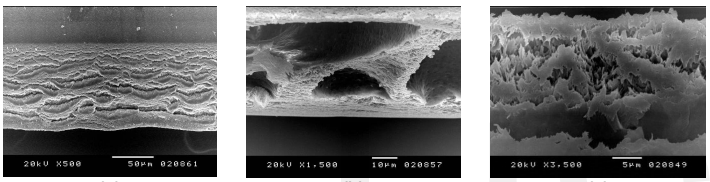
(a) (b) (c)

Fig. 6 SEM micrographs of CDA membranes surfaces fabricated by exposure to humid air flow followed by phase inversion, solution composition: (a) CDA 22 g in DMF 88 mL, (b) CDA 22 g in D 138 mL, (c) CDA 10 g in (CF 88 mL + D 50 mL)

At comparing the two previous solvents with the third one, Fig. (6c), in which CF functioned as solvent together with D, presented at a much lower concentration, and which had proven to be a less suitable membrane in the formation of pores/scaffolds, resulted in almost the same morphology as Fig. (6b), since the good effect of the casting solution being dilute, nullified the first negative effect of the presence of CF in the casting solution mixture. Accordingly, the two latter micrographs were more or less similar, in that they contain mini- and micro- pores, as regards the surface morphology; however, some scaffolds are apparent from the pores in Fig. (6b). It is noteworthy that microorganisms are visible on the surface and which cover many pores and these lend promise for the attachment of human cells and their proliferation onto the surface. To this end, the present observations emphasizes that the morphology of the surface is largely dependent on the type of solvent, which has been stated by Duarte et al [47], and to some extent on the concentration of the CDA solution, especially that clusters of micro-particles are only apparent in the more concentrated solution (see Fig. 6a). It is worth noting that 60 minutes of exposure to humid air flow, seems to be longer than necessary, since the surfaces tended to dry rapidly and retain the solvents inside, which if cyto-toxic should lead to cell death in the bioreactor if not totally removed. According to Liu et al [35], when using organic solvents to create pores within the scaffolds, the removal of the solvent remains a problem and forms a potential source of toxicity for cells and could reduce the ability of cells to form new tissue *in vivo*. The

extensive use of solvents (some of which are toxic) may present a difficulty, as any residuals of the solvent would hinder the cell attachment and proliferation onto the scaffold [48],[49]. Nevertheless, low toxicity solvents can be used and residues brought down to acceptable levels for application [35],[50].

The effect of the same factor is noticed on examining the two Figs. (7a and b) in which the cross-sections of the two aforementioned membranes (Figs. 7a and b) are shown and in which DMF and D were used as solvents, respectively. Figure (7a) indicates the non-porous nature of the membrane from DMF as solvent. However, some lateral voids are apparent but which are small and not deep inside the membrane matrix, causing the latter to be dense and only of about 10% porosity. On the other hand, the membrane formed from D as solvent and which is shown in Fig. (7b) clarifies the highly porous texture of the matrix, as well as the scaffolds holding the membrane intact. In addition to the macro-voids which should assist in future cell seeding leading to skin tissue formation.



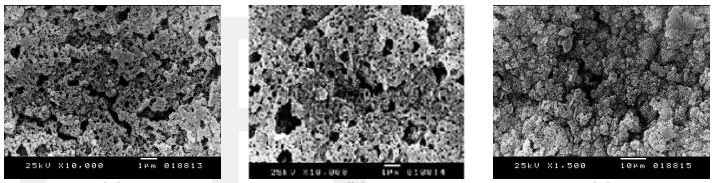
(a) (b) (c)

Fig. 7 SEM micrographs of CDA membranes cross-sections fabricated by exposure to humid air flow followed by phase inversion, solution composition: (a) CDA 22 g in DMF 88 mL, (b) CDA 22 g in D 138 mL, (c) CDA 10 g in (CF 88 mL + D 50 mL)

Comparing Figs. (7b and c) in which D alone, and D plus CF were used as solvents, respectively, for the CDA polymer, emphasizes the effect of solvent on the membrane morphology. It is clear that while D alone causes the membrane to be highly porous with plenty of scaffolds and macro-voids in its matrix, on the other hand the presence of CF along with D results in a much less porous matrix, despite that macro-voids are present but to less extent. However, it is noticed that before membrane sputtering with gold (an essential step prior to scanning with the electron microscope) the membrane should be cut (sliced) under liquefied nitrogen [51], in order to avoid the sliding of one layer onto the other as is shown in Fig. (7c). Accordingly, it may be concluded that D is best as solvent for CDA, followed by D plus CF blend then DMF being the least efficient in forming pores/scaffolds in the membrane matrix.

3.4 Effect of time of exposure to humid air flow

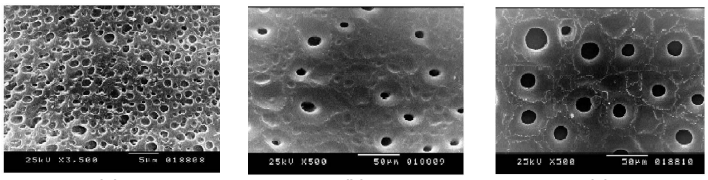
The effect of time of exposure to humid air flow on the surface microstructure of CS/CDA blend membrane fabricated under the conditions mentioned in the figure caption is shown on comparing Figs. (8a, b, and c) in which the membranes were subjected to humid air flow for 10, 20, and 30 minutes respectively, taking into consideration the difference in degree of magnification. Inspecting the three figures reveals the better surface structure of the membrane exposed for 30 minutes to humid air flow, in spite that the degree of magnification (1,500X) is much lower relative to that of the other two Figs. (8a and b) (10,000X), in that the surface contains plenty micro- and mini- pores homogeneously distributed within the polymer matrix with a large density surface area of solid clusters which may have been probably formed due to partial surface drying due to solvent evaporation on prolonged treatment. On the other hand, the membrane exposed for 10 minutes (Fig. 8a) contains numerous nano-pores more or less homogeneously distributed and which are connected to each other by nano-particles of the polymer blend. Moreover, the surface seems to have been smooth since no clusters are visible. Finally, Fig. (8b) which was exposed for 20 minutes gives an intermediate porous structure to the other two cases emphasizing our observation that the more time of exposure to humid air flow the more the number of nano- and micro- particles and the less the membrane surface density providing limited time of exposure. Therefore, it could be concluded that prolonged exposure to humid air flow is not required, which on the other hand will present a saving in energy.



(a) (b) (c)

Fig. 8 SEM micrographs of CS/CDA membranes surfaces (CS:CDA = 1:1 w/w in FA:DMF = 1:1 b.v.) by exposure to humid air flow followed by phase inversion: Time of exposure (a) 10 minutes (10,000X), (b) 20 minutes (10,000X), (c) 30 minutes (1,500X)

Figs. (9a, b, and c) represents the surface morphology of the same three aforementioned membranes at much lower magnification of 3,500, 500, and 500, respectively. It is obvious that the pores became wider by increasing the time of exposure to humid air flow. In addition, corrugated lines are present which may be due to initiation of cleavage of portions of the membrane matrix from each other due to increased time of exposure to humid air which initiates rupture of the membrane matrix due to increased thickness rather than the formation of pores. Thus, 30 minutes as well as 20 minutes are undesirable in the case of CDA membrane.



(a) (b) (c)

Fig. 9 SEM micrographs of CS/CDA membranes surfaces (CS:CDA = 1:1 w/w in FA:DMF = 1:1 b.v.) by exposure to humid air flow followed by phase inversion, time of exposure (a) 10 minutes (3,500X), (b) 20 minutes (500X), (c) 30 minutes (500X)

4 Conclusions

This study confirms that CS is a suitable membrane material which can form scaffolds/pores readily and the blend of CDA with CS can form smooth-surfaced membranes with a high population of micro- and mini- pores under precise conditions. The CS/CDA blend membrane surface is less smooth than the CDA membrane. Also, thinner membranes are obtained from dilute polymer solutions being more suitable for skin tissue formation than thicker ones, where the type of solvents used in dissolving the polymer largely influences the formation of scaffolds/pores.

Our work proves that 30 minutes of exposure to humid air flow leads to the formation of a multiplicity of interconnected scaffolds/pores compared to 10 minutes. In additional, CS is a very versatile membrane material due to its biocompatibility, biodegradability, availability and low cost. Techniques by which scaffolds and pores are formed are simple, novel, of low cost and efficient. The proliferation of microorganisms on top and within the pores and scaffolds of some membranes proves that cell seeding will be possible in a suitable bioreactor.

References

[1] I-Y. Kim, S-J. Seo, H-S. Moon, M-K. Yoo, I-Y. Park, B-C. Kim, and C-S. Cho, "Chitosan and its derivatives for tissue engineering applications", *Biotechnology Advances*, vol. 26, no. 1, pp. 1–21, Jan/Feb 2008.

[2] P.X. Ma, "Scaffolds for tissue fabrication", *Materials Today*, vol. 7, no. 5, pp. 30–40, May 2004.

[3] A.G. Mikos, A.J. Thorsen, L.A. Czerwonka, Y. Bao, R. Langer, D.N. Winslow, and J.P. Vacanti, "Preparation and characterization of poly(L-lactic acid) foams", *Polymer*, vol. 35, no. 5, pp. 1068–1077, Mar. 1994.

[4] D.W. Hutmacher, "Scaffolds in tissue engineering bone and cartilage", *Biomaterials*, vol. 21, no. 24, pp. 2529–2543, Dec. 2000.

[5] E. Sachlos, and J.T. Czernuszka, "Making tissue engineering scaffolds work. Review on the application of solid freeform fabrication technology to the production of tissue engineering scaffolds", *European Cells and Materials*, vol. 5, pp. 29–40, Jun. 2003.

[6] A.G. Mikos, Y. Bao, L.G. Cima, D.E. Ingber, J.P. Vacanti, R. Langer, "Preparation of poly (glycolic acid) bonded fibres structures for cell attachment and transplantation", *Journal of Biomedical Materials Research*, vol. 27, no. 2, pp. 183–189, Feb. 1993b.

[7] A.G. Mikos, G. Sarakinos, J.P. Vacanti, R. Langer, and L.G. Cima, "Biocompatible polymer membranes and methods of preparation of three dimensional membrane structures", *US Patent Number 5514378*, May 1996.

[8] H. Lo, M.S. Ponticiello, and K.W. Leong, "Fabrication of controlled release biodegradable foams by phase separation", *Tissue Engineering*, vol. 1, no. 1, pp. 15–28, Mar. 1995.

[9] D.J. Mooney, D.F. Baldwin, N.P. Suh, J.P. Vacanti, and R. Langer, "Novel approach to fabricate porous sponges of poly(D,L-lactic co-glycolic acid) without the use of organic solvents", *Biomaterials*, vol. 17, no. 14, pp. 1417–1422, Jul. 1996.

[10] Y.Y. Hsu, J.D. Gresser, D.J. Trantolo, C.M. Lyons, P.R.J. Gangadharam, and D.L. Wise, "Effect of polymer foam morphology and density on kinetics of *in vitro* controlled release of isoniazid from compressed foam matrices", *Journal of Biomedical Materials Research*, vol. 35, no. 1, pp. 107–116, Apr. 1997.

[11] M. Reuber, L.S. Yu, and W.J. Kolff, "Effect of processing temperature on the properties of polyurethane and comparison of vacuum forming and solution casting to make artificial hearts", *Artificial Organs*, vol. 11, pp. 323–332, 1987.

[12] J.P. Schmitz, and J.O. Hollinger, "A preliminary study of the osteogenic potential of a biodegradable alloplastic-osteoinductive alloimplant", *Clinical Orthopaedics and Related Research*, vol. 237, pp. 245–255, Dec. 1988.

[13] K. Whang, C.K. Thomas, G. Nuber, and K.E. Healy, "A novel method to fabricate bioadsorbable scaffolds", *Polymer*, vol. 36, no. 4, pp. 837–842, 1995.

[14] R.C. Thompson, M.J. Yaszembksi, J.M. Powers, T.P. Harrigan, and A.G. Mikos, "Poly(a-hydroxy ester)/short fiber hydroxiapatite composite foams for orthopedic applications", *Materials Research Society Symposium Proceedings*, Pittsburgh, vol. 394, pp. 25–30, 1995.

[15] I.V. Yannas, J.F. Burke, P.I. Gordon, C. Huang, and R.H. Rubenstein, "Design of an artificial skin, II. Control of chemical composition", *Journal of Biomedical Materials Research*, vol. 14, no. 2, pp. 107–132, Mar. 1980.

[16] Y. Wan, Y. Ya Fang, H. Wu, and X. Cao, "Porous polylactide/chitosan scaffolds for tissue engineering", *Journal of Biomedical Materials Research*, vol. 80A, pp. 776 –789, 2007.

[17] W.G. Malette, J. Quigley, and E.D., Adickes, "Chitosan effect in vascular surgery, tissue culture and tissue regeneration", *Chitin in Nature and Technology*, (R. Muzzarelli, C. Jeuniaux, and G.W. Gooday, eds), Plenum Press, New York, pp. 435–442, 1986.

[18] K. Mizuno, K. Yamamura, K. Yano, T. Osada, S. Saeki, N. Takimoto, T. Sakurai, and Y. Nimura, "Effect of chitosan film containing basic fibroblast growth factor on wound healing in genetically diabetic mice", *Journal of Biomedical Materials Research*, vol. 64, no. 1, pp. 177–181, Jan. 2003.

[19] G.I. Howling, P.W. Dettmar, P.A. Goddard, F.C. Hampson, M. Dornish, and E.J. Wood, "The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes *in vitro*", *Biomaterials*, vol. 22, no. 22, pp. 2959–2966, Nov. 2001.

[20] X.L. Yan, E. Khor, and L.Y. Lim, "PEC films prepared from Chitosan-Alginate coacervates", *Chemical and Pharmaceutical Bulletin*, vol. 48, no. 7, pp. 941–946, Jul. 2000.

[21] L.S. Wang, E. Khor, A. Wee, and L.Y. Lim, "Chitosan–Alginate PEC membrane as a wound healing: Assessment of incisional wound dressing", *Journal of Biomedical Materials Research*, vol. 63, no. 5, pp. 610–618, 2002.

[22] L. Ma, C. Gao, Z. Mao, J. Zhou, J. Shen, X. Hu, and C. Han, "Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering", *Biomaterials*, vol. 24, no. 26, pp. 4833–4841, May 2003.

[23] P.B. Malafaya, T.C. Santos, M. van Griensven, and R.L. Reis, "Morphology, mechanical characterization and *in vivo* neo-vascularization of chitosan particle aggregated scaffolds architectures", *Biomaterials*, vol. 29, no. 29, pp. 3914–3926, Oct. 2008.

[24] Y. Jiao, Z. Liu, and C. Zhou, "Fabrication and characterization of PLLA–chitosan hybrid scaffolds with improved cell compatibility", *Journal of Biomedical Materials Research*, vol. 80A, no. 4, pp. 820–825, Mar. 2007.

[25] A.R.C. Duarte, J.F. Mano, and R.L. Reis, "Novel 3D scaffolds of chitosan–PLLA blends for tissue engineering applications: Preparation and characterization", *Journal of Supercritical Fluids*, vol. 54, no. 3, pp. 282–289, Sep. 2010.

[26] Y-K. Twu, I-T. Chang, and C-C. Ping, "Preparation of novel chitosan scaffolds by electrochemical process", *Carbohydrate Polymers*, vol. 62, no. 2, pp. 113–119, Nov. 2005.

[27] J.S. Mao, L.G. Zhao, Y.J. Yin, and K.D. Yao, "Structure and properties of bilayer chitosan–gelatin scaffolds", *Biomaterials*, vol. 24, no. 6, pp. 1067–1074, Mar. 2003.

[28] R. Jayakumar, R. Ramachandran, P.T.S. Kumar, V.V. DIvyarani, S. Srinivasan, K.P. Chennazhi, H. Tamura, and S.V. Nair, "Fabrication of chitin–chitosan/nano ZrO_2 composite scaffolds for tissue engineering applications", *International Journal of Biological Macromolecules*, vol. 49, no. 3, pp. 274–280, Oct. 2011.

[29] Y. Li, Y. Wang, D. Wu, K. Zhang, and Q. Hu, "A facile approach to construct three-dimensional oriented chitosan scaffolds with in-situ precipitation method", *Carbohydrate Polymers*, vol. 80, no. 2, pp. 408–412, Apr. 2010.

[30] N. Boucard, C. Viton, D. Agay, E. Mari, T. Roger, Y. Chancerelle, and A. Domard, "The use of physical hydrogels of chitosan for skin regeneration following third-degree burns", *Biomaterials*, vol. 28, no. 24, 3478–3488. Aug. 2007.

[31] M.S. Kim, S.J. Park, B.K. Gu, and C-H. Kim, "Interconnecting pores of chitosan scaffold with basic fibroblast growth factor modulate biological activity on human mesenchymal stem cells", *Carbohydrate Polymers*, vol. 87, no. 4, pp. 2683–2689, Mar. 2012.

[32] N. Sawaguchi, T. Majima, T. Funakoshi, K. Shimode, K. Harada, A. Minami, and S-I. Nishimura, "Effect of cyclic three-dimensional strain on cell proliferation and collagen synthesis of fibroblast-seeded chitosan-hyaluronan hybrid polymer fiber", *Journal of Orthopaedic Science*, vol. 15, no. 4, pp. 569–577, Jul. 2010.

[33] Y-C. Kuo, and Y-R. Hsu, "Tissue-engineered polyethylene oxide/chitosan scaffolds as potential substitutes for articular cartilage", *Journal of Biomedical Materials Research*, vol. 91, no. 1, pp. 277–287, Oct. 2009.

[34] N. Maruyama, T. Koito, J. Nishida, T. Sawadaishi, X. Cieren, K. Ijiro, O. Karthaus, and M. Shimomura, "Mesoscopic patterns of molecular aggregates on solid substrates", *Thin Solid Films*, vol. 327, no. 1, pp. 854–856, Aug. 1998.

[35] C. Liu, Z. Xia, and J.T. Czernuszka, "Design and development of three-dimensional scaffolds for tissue engineering", *Chemical Engineering Research and Design*, vol. 85, no. 7, pp. 1051–1064, Jul. 2007.

[36] M.M. Naim, "Novel chemically-modified cellulose membranes for the artificial kidney", *The Transactions of the Egyptian Society of Chemical Engineers*, Paper presented to the First Egyptian Congress of Chemical Engineering, vol. 6, no. 1, pp. 33–39, Jun. 1980.

[37] M.M. Naim, "Novel deacetylated cellulose acetate dialysis membranes", *The Bulletin of the Faculty of Engineering*, Faculty of Engineering Press, Alexandria University, vol. XXV, pp. 147–165, 1986.

[38] M.M. Naim, and Y.A. El Tawil, "Novel regenerated cellulose membrane suitable for dialysis – determination of diffusion coefficient of sodium chloride through membrane", *Journal of Engineering Sciences,* King Saud University, vol. 8, no. 2, pp. 127–131, 1982.

[39] C.W. Saltonstall, W.M. King, and D.L. Hoernschemeyer, "A new method for measuring diffusion coefficients of electrolytes in nonionic membranes", *Desalination*, vol. 4, no. 3, pp. 309–327, 1968.

[40] H.Ç. Arca, and S. Şenel, "Chitosan based systems for tissue engineering Part 1: Hard tissues", *FABAD Journal of Pharmaceutical Sciences*, vol. 33, no. 1, pp. 35–49, Mar. 2008.

[41] C.K. Lim, and A.S. Halim, "Biomedical-grade chitosan in wound management and its biocompatibility *in vitro*", *Biopolymers*, Magdy Elnashar (Ed.), InTech, Malaysia, pp. 19–36, Sep. 2010.

[42] R. Jayakumar, M. Prabaharan, P.T. Sudheesh Kumar, S.V. Nair, and H. Tamura, "Biomaterials based on chitin and chitosan in wound dressing applications", *Biotechnology Advances*, vol. 29, no. 3, pp. 322–337, May/Jun. 2011.

[43] M.H. Stenzel, C. Barner-Kowollik, and T.P. Davis, "Formation of honeycomb-structured, porous films via breath figures with different polymer architectures", *Journal of Polymer Science Part A: Polymer Chemistry*, vol. 44, no. 8, pp. 2363–2375, Apr. 2006.

[44] M.M. Naim, "Novel regenerated cellulose membrane suitable for haemodialysis", *The Transactions of the Egyptian Society of Chemical Engineers,* Paper presented to the First Egyptian Congress of Chemical Engineering, vol. 6, no. 1, pp. 19–32, Jun. 1980.

[45] D. Bartis, and J. Pongrácz, "Three dimensional tissue cultures and tissue engineering", *University of Pécs*, 2011.

[46] S.K. Purna, and M. Babu, "Collagen based dressings- A review", *Burns*, vol. 26, no. 1, pp. 54–62, Feb. 2000.

[47] A.R.C. Duarte, J.F. Mano, and R.L. Reis, "The role of organic solvent on the preparation of chitosan scaffolds by supercritical assisted phase inversion", *The Journal of Supercritical Fluids*, vol. 72, pp. 326–332, Dec. 2012.

[48] G.P. Chen, T. Ushida, and T. Tateishi, "Development of biodegradable porous scaffolds for tissue engineering", *Materials Science and Engineering: C*, vol. 17, no. 1–2, pp. 63–69, Nov. 2001.

[49] D.J. Mooney, D.F. Baldwin, N.P. Suh, J.P. Vacanti, and R. Langer, "Novel approach to fabricate porous sponges of poly(D, L-lactic-co-glycolic acid) without the use of organic solvents", *Biomaterials*, vol. 17, no. 14, pp. 1417–1422, Jul. 1996.

[50] Y. Cao, G. Mitchell, A. Messina, L. Price, E. Thompson, A. Penington, W. Morrison, A. O'Connor, G. Stevens, and J. Cooper-White, "The influence of architecture on degradation and tissue ingrowth into three-dimensional poly(lactic-co-glycolic acid) scaffolds *in vitro* and *in vivo*", *Biomaterials*, vol. 27, no. 14, pp. 2854–2864, May 2006.

[51] N. Nadi, City of Scientific Research and Technological Applications, Alexandria, Egypt, *Personal communication*, Jan. 2014.