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Cryogenic Designing of Biocompatible Blends of Polyvinyl alcohol and Starch with Macroporous Architecture

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The present paper discusses synthesis, characterization, and blood compatibility studies of macroporous cryogels of PVA and starch. Biocompatible spongy porous hydrogels of polyvinyl alcohol–starch have been synthesized by repeated freezing–thawing methods and characterized by Infra red (FTIR) and environmental scanning electron microscopy (ESEM) techniques, respectively, to gain insights for structural and morphological features. The FTIR analysis of prepared cryogels indicated that starch was introduced into the network of cryogel possibly via formation of hydrogen bonds between the PVA and starch clusters. The "cryogels" were evaluated for their water uptake potentials and influence of various factors such as chemical architecture of the spongy hydrogels, pH and temperature of the swelling bath were investigated on the degree of water sorption by the cryogels. The hydrogels were also swollen in salt solutions and various simulated biological fluids. The biocompatibility of the prepared cryogels was judged by *in vitro* methods of blood–clot formation viz. percent haemolysis and protein (BSA) adsorption. The cryogels were also studied for their pores morphology and percent porosity and the effect of chemical composition on the extent of porosity was also investigated.

Keywords: Biocompatibility, freeze-thaw, cryogel, macroporous

1 Introduction

The incidences of organ and tissue loss or failure are increasing steadily, whereas immune rejection and the number of donors limit the traditional surgical treatment of implantation of a healthy organ from a donor. As an application of tissue engineering, the use of cell transplantation is now being investigated as an alternative therapeutic strategy for tissue repair and organ replacement (1). In culturing cells, shape of the scaffold a temporary substrate to allow growth specialization of the cell culture, plays an important role (2). Polymeric scaffolds must be porous enough to allow a high density of cells to be seeded, yet also possesses sufficient mechanical stability and a well defined network of interconnected pores permit growth into the implanted structure. Thus, macroporous polymers meet all the desired qualities and, therefore, have been extensively utilized in tissue engineering (3).

The intrinsic properties such as mechanical (4) or thermal (5) and structural features like pore size, porosity, pore morphology etc. play a vital role in shaping suitability of the macroporous materials for a specific application. Furthermore, biodegradation of the macroporous material after the intended time period is another essential property of polymer matrix considered for tissue engineering applications. However, a potential disadvantage of biodegradation is the eventual toxicity following absorption of the degraded products which may be overcome by proper choice of the natural polymer.

Polysaccharide based biodegradable matrices are of interest since the degradation of a natural products like starch occurs naturally in the human body (6) and, therefore, the polymer matrices based on starch and its derivatives may prove to be relevant materials for biomedical application. By integrating biological entities with synthetic hydrogels novel systems can be created that synergistically combine well-evolved biological mechanisms, such as his affinity and specificity of binding, with tailorable, hydrogel properties (e.g. mechanical stability and environmental-responsing properties). Thus, adopting the above strategy the present work aims at designing a hybrid polymer matrix of starch and polyvinyl alcohol (PVA) which could offer desirable properties like porosity, biocompatibility, water-sorption and biodegradability and therefore, could be employed in tissue engineering and related applications.

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Starch is composed of two distinct fractions: amylose is the fraction containing about 4000 glucose units, and amylopactin is the branched fraction containing about 100000 glucose units. Starch and crosslinked starch obtained by treatment with reagents like epichlorohydrin, adipic anhydride etc are widely and safely used with the agreement of the food and drug administration in the food (thickener, enhancer of organolaptic properties, textile modifier, etc). Starch is naturally hydrolyzed by several amylolytic enzymes like α -amylase which is an endo enzyme specific to α -(1,4)–D glucopyranosidic bonds located within polyglucose chains. The degradation products of starch amylolysis are mainly composed of oligosaccharaides, dextrins and maltose (7). Among various synthetic hydrophilic polymers used in the preparation of hydrogels, polyvinyl alcohol, hence referred to as PVA, owes a prime position in biomaterials science because of its inherent non-toxicity, non-carcinogenicity, good biocompatibility and desirable physical properties such as rubbery, or elastic nature and high degree of swelling in aqueous solutions (8). PVA is a linear polymer and can be easily crosslinked with irradiation (9) or bifunctional group-containing chemical agents such as glutaraldehyde (10), boric acid (11), hexamethylene diisocyanat (12), etc. Although a great deal of work has been done in the production of crosslinked starch by various techniques, however, traditional methods of crosslinking suffer from several disadvantage such as lack of precise control over crosslinking, toxic effects of crosslinker, loss in bioactivity of entrapped agent etc. It was only after the novel efforts of several research groups who developed a physical crosslinking method for synthesis of PVA hydrogels by repeated freezing thawing process of a moderately concentrated PVA aqueous solution. The hydrogels so produced were porous, spongy, rubbery and elastic and displayed good mechanical strength.

Li and coworkers (13) followed freeze thawing procedure for preparing PVA nanoparticles and studied peptide delivery from the loaded nanoparticles. Jio and Minoura (14) studied the swelling behavior of a blend hydrogel made of poly(allylgn anidino–co-allylamine) and PVA by the freeze thaw method and observed the influence of pH on water sorption capacity. Bajpai and Saini (15) prepared the hydrogel of PVA and egg albumin by the repeated freeze thaw process and evaluated their water sorption capacity and biocompatibility. The same authors (16) synthesized the polymeric blend of starch and carboxymethyl cellulose and studied their enzymatic degradation. Thus, being motivated by the vital role of biocompatible porous macrostructures in biomedical applications, the objectives of the present work include preparation of polymeric blends of starch with PVA and their characterization by FTIR, and ESEM techniques, study of water sorption kinetics of the prepared blends and evaluation of *in vitro* biocompatibility of the matrices.

2 Experimental

2.1 Preparation of Polymer Blends

The freeze thaw method was adopted for preparation of blend hydrogels of starch and PVA as described elsewhere (17–19). In a typical experiment, definite amounts of starch and PVA were dissolved into distilled water in a petri-dish and kept at –20°C for 24 h. The frozen gel is then thawed for 1 h at room temperature and again –20°C for freezing. Such freezing-thawing cycles were repeated at least three times so that the entie mass converted into a soft, spongy, white blend. The blend so prepared was purified by equilibrating it in distilled water for 72 h, so that all unreacted chemicals were leached out. The swollen blend was cut into smaller discs and dried at room temperature for a week. The dried blend pieces were stored in airtight polyethylene bags.

2.2 Characterization of Blends

2.2.1. *FTIR spectral analysis*

The infrared spectral analysis of the prepared hydrogels was performed on an FTIR spectrophotometer (FTIR-8400S, Shimadzu spectrophotometer) by recording the IR spectra of a dry thin film of the blend.

2.3 ESEM Studies

The morphological features of the cryogels were investigated by recording their Scanning Electron Micrographs (STEREO SCAN, 430, Leica, SEM, USA).

2.4 Water Sorption Capacity

Water intake capacity of blend was determined by a conventional gravimetric procedure as reported in the literature (20). In brief, a pre-weighed dry piece of blend is immersed into distilled water at room temperature, taken out at predetermined time intervals, gently pressed between two filter papers to remove excess water and finally weighed on a sensitive balance (APX-203 Denver, Germany). The swelling ratio is calculated with the help of following equation,

$$\text{Swelling Ratio} = \frac{\text{Weight of swollen gel (Ws)}}{\text{Weight of dry gel (Wd)}} \qquad (1)$$

2.5 Determination of Porosity

The apparent porosity of a porous scaffold can influence in mechanical strength, permeability and presence of strutural defects (A). The porosity was determined by the method reported in literature (B). In brief, the known volume and weights of the samples noted as Vo and Wo, respectively, After that, samples were immersed into the dehydrated alcohol for 48 h till absorbing dehydrated alcohol saturated

the samples, The weight gain by the sample is measured as W1. Finally, the porosity (p) of the open pores in the blend were evaluted being formula given below,

$$P = \frac{W_1 - W_0}{pV_0} \quad (2)$$

Where P is density of the dehydrated alcohol.

2.6 Blood Compatibility

In order to evaluate the suitability of a polymer materix to biomedical applications, particularly as artificial implant or for *in vivo* use, the biomaterial must meet certain criteria prior to its actual realization. Thus, to judge the blood compatible nature of the material, certain test procedures have been establisehd for determianton of *in vitro* blood compatibility of the polymer material as discussed in the following section.

2.7 Protein Adsorption

The foremost event occurring at the interface of the blood- material contact is the adsorption of plasma proteins (bovine serum albumin, fibrinogen etc.) which subsequently influences the adhesion of leukocytes, macrophages or platelets and ultimately leads to fibrous encapsulation. Thus, the adsorption of proteins could be one of the determinants of biocompatible nature of the material. The adsorption of proteins was studied as described below.

A known volume of protein solution of definite concentration is mildly shaken with the polymer blend for a definite time period and the reaming concentration of protein is monitored in the solution spetrophotometrically. The amount of the adsorbed protein is calculated with the help of the following mass balance equation,

$$\text{Adsorbed amount (mg g}^{-1}) = \frac{(C_o - C_a)\,V}{A} \quad (3)$$

Where, Co and Ca being the concentrations of protein solution (mg per mL) before and after adsorption, respectively, V is the volume of the protein solution and A is the surface area of the adsorbent.

2.8 Clot Formation Test

The antithrombogenic potential of the blends surface was judged by the blood clot formation test as described elsewhere (21). In brief, the starch blends are equilibrated with saline water (0.9% w/v NaCl) for 72 h in a constant temperature bath and to these swollen blends are added 0.5 mL of acid citrate dextrose (ACD) blood followed by the addition of 0.03 mL of $CaCl_2$ solution (4M) to start the thrombus formation. The reaction is stopped by adding 4.0 mL of deionized water and the thrombus formed is separated by soaking in water for 10 min at room temperature and then fixed in a 36% formaldehyde solution (2.0 mL) for another 10 min. The fixed clot is placed in water for 10 min and weight after drying.

2.9 Hemolysis Assay

Hemolysis experiments were performed on the surfaces of the prepared blends as reported elsewhere (22). In a typical experiment, a dry blends film is equilibrated in normal saline water (0.9% NaCl solution) for 24 h at 37°C and human ACD blood (0.25 mL) was added onto the blend films. After 20 min, 20 mL of saline is added on the surface to stop hemolysis and the sample is incubated for 60 min at 37°C. Positive and negative controls are obtained by adding 0.025 mL of human ACD blood and saline solution, respectively to 2.0 ml of distilled water. Incubated samples are centrifuged for 45 min, the supernatant is taken and its absorbance is recorded on a spectrophotometer at 545 nm. The percent hemolysis may be calculated using the following relationship,

$$\%\,\text{Hemolysis} = \frac{\text{A test sample} - \text{A}(-)\,\text{control}}{\text{A}(+)\text{control} - \text{A}(-)\text{control}} \quad (4)$$

Where A = Absorbance.

3 Results and Discussion

3.1 Mechanism of Cryogel Formation

A mechanism of hydrogel preparation involves physical crosslinking due to crystallite formation. This method addresses toxicity issues because it does not require the presence of a crosslinking agent. Such physically crosslinked materials exhibit higher mechanical strength than PVA blends crosslinked by chemical or irradiative techniques because the mechanical load can be distributed along the crystallites of the three-dimensional structure (23). Although formation of an elastic blend either upon standing an aqueous solution of PVA at room temperature or successively freezing-thawing a moderately concentrated PVA solution is not new concept (24), however, a molecular explanation for this phenomenon are not yet available. Three basic models including hydrogen bonding, polymer crystallites formation and liquid-liquid phase separation have been suggested to explain the mechanisms involved in the blend formation.

In the present study, since a mixture of PVA and starch solution was taken, the above three models may be considered to become operative simultaneously, thus yielding a highly elastic blend. The reason for this assumption is that starch is a multifunctional biopolymer and its presence in PVA solution will enhance the processes of hydrogen bond making, polymer crystallite formation and phase separation, respectively. The porous nature of blends produced by freezing-thawing method may be explained by the fact that whereas the freezing of a PVA-starch mixture results

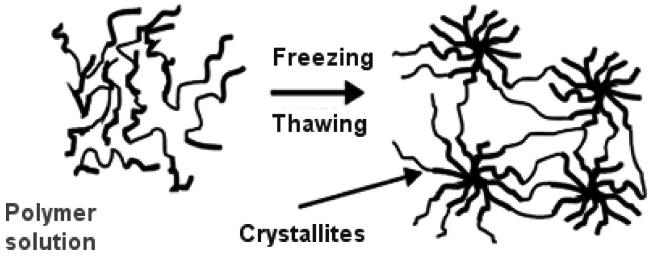

Fig. 1. Mechanism of Cryogel formation.

in the formation of ice crystal domains within the polymer mixture matrix, the thawing process results in melting of the ice crystals, thus leaving wide pores in the blends. A repeated performance of the processes widens the pore sizes and thus enhances the porous nature of the blends. The formation of porous network due to freezing-thawing method is modeled in Figure 1.

3.2 FTIR Analysis

The FTIR spectra of the blend composed of PVA and Starch is depicted in Figure 2. The FTIR spectra clearly shows a broad band around 3583 cm^{-1} which is typical of bonded O—H stretching of alcoholic hydroxyls of starch and PVA. The spectra shows prominent peaks at 2924 cm^{-1} due to =CH stretching of methylene group, 1795 cm^{-1} due to overtone or combination bands, 1483 cm^{-1} due to sp3-CH bending, and 1151cm^{-1} due to C-O stretching of alcohol.

3.3 Scanning Electron Microscopy

The morphological features of the proposed cryogel have been studied by SEM analysis. In order to demonstrate how the freeze-thaw method develops the formation of pores over the cryogel surfaces, a blend of PVA- starch was also prepared by simple solution cast method and the SEM images of both the types of blends were compared. The SEM images of simple and cryogel blends of PVA and starch are shown in Figure 3(a) and (b), respectively. The image (a) clearly shows that both the PVA and starch form a homogeneous blend having no porosity, unevenness, cracks or voids on the surfaces. On the other hand, the image (b) presents a porous morphology with pore sizes varying in the range 25 to 50 μ m. In this way, a clear distinction between the two images provides an experimental evidence for the proposed formation of crystallites during the freezing-thawing method.

3.4 Water Sorption Measurement

3.4.1. *Effect of PVA*

3.4.1.1. *Effect of PVA/starch.* As PVA and starch are the main constituent of the cryogel, their varying weight fraction in the hydrogel exert a significant influence on the water

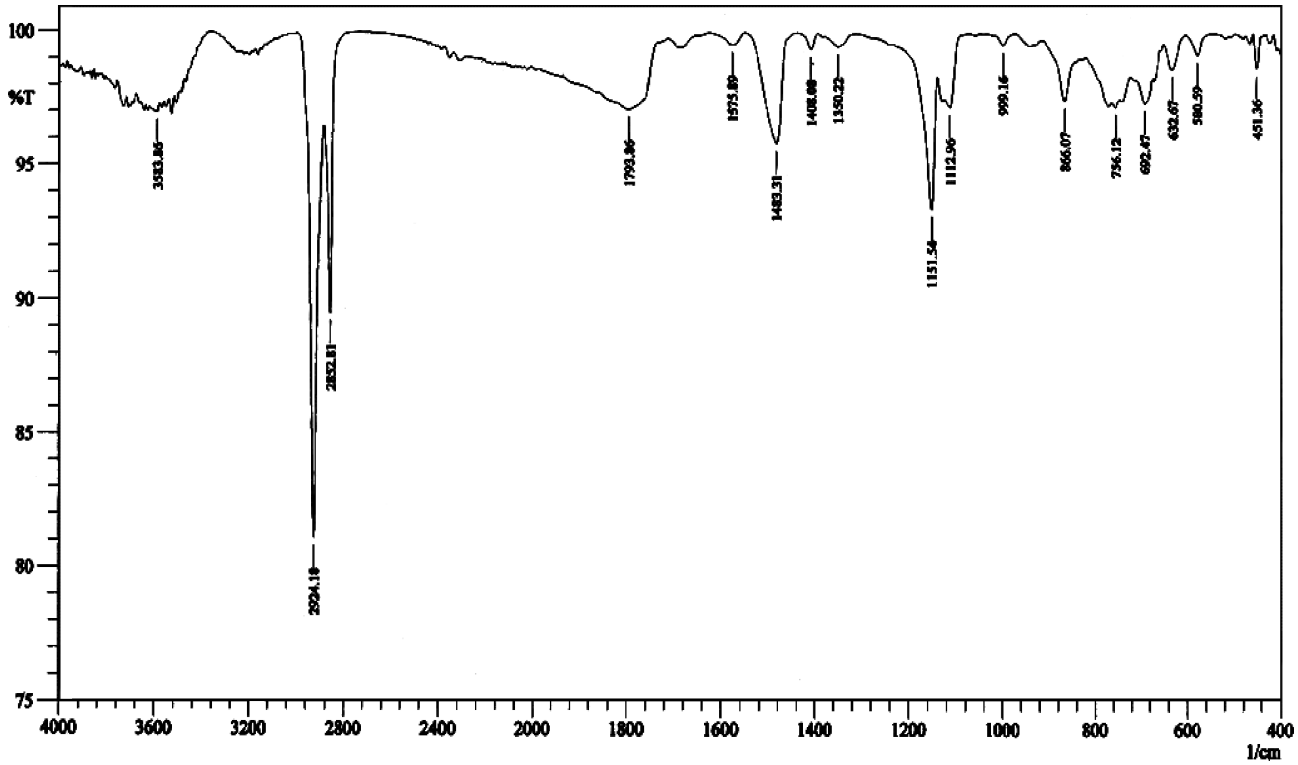

Fig. 2. FTIR Spectra of PVA and starch.

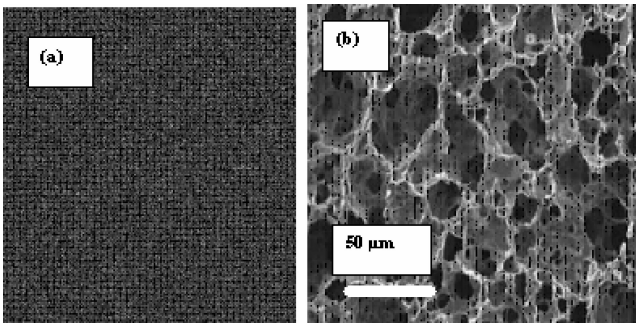

Fig. 3. The SEM images of PVA and starch blends.

sorption capacity, as well as other allied properties. Thus the effect of PVA/starch on the swelling ratio has been investigated by varying the wt. fraction of PVA on the range to 20 to 42.8 (%w/w) or that of starch in the range (57.2 to 80), respectively. The results are summarized in Figure 4 which clearly indicate that swelling ratio initially increase with increasing wt. fraction of PVA from 20 to 33.3, while beyond 33.3, the swelling ratio falls. The reason for the observed increase in swelling ration could be attributed to the fact that PVA being a hydrophilic polymer and its increasing amount in the cryogel results in increased hydrophilicity of the gel which brings about an increase in the water sorption capacity. However, beyond 33.3%(w/w) content of PVA, the density of network chains become so high that penetration of water molecules into the cryogel network becomes rather difficult and swelling ratio decreases. Similar type of observations have also been noticed by other workers (25, 26).

In a similar way, when wt% of starch increases from 57.2 to 66.7, an increase in swelling ratio is noticed while beyond 66.7 a decrease in swelling ratio is observed. The reason for the observed increase in swelling ratio is due to the fact that like PVA, starch is also a nonionic but hydrophilic biopolymer and, therefore, its increasing content in the cryogel brings about an increase in swelling ratio.

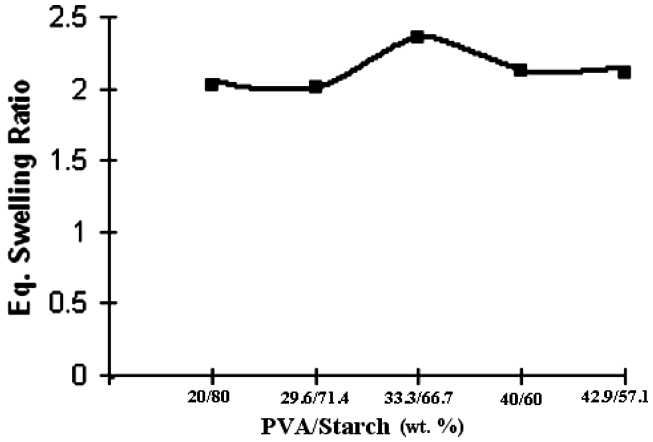

Fig. 4. Effect of wt. fraction of PVA/starch on swelling ratio.

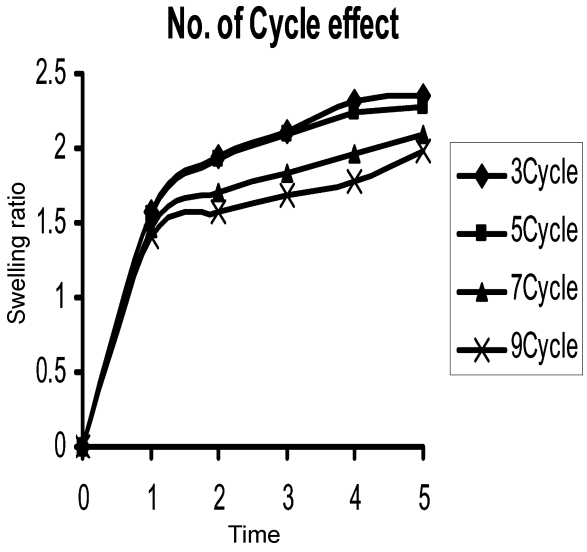

Fig. 5. Effect of freeze-thaw cycle on swelling ratio.

However, beyond 66.7% (w/w) of starch the water sorption capacity falls which may be due to the compact nature of the cryogel network that restrains the inclusion of water molecules in the network and swelling ratio decreases.

3.4.2. *Effect of freeze-thaw cycles (FTC)*

The effect of number of repeated freeze-thaw cycle on the swelling ratio of cryogel has been studied and shown in Figure 5. The results clearly reveal that the extent of swelling ratio decreases with increasing number of FTC. The observed results may be attributed to the fact that in the case of 3rd cycle, the cryogel does not have large crystalline regions and therefore, the chains of PVA and starch keep on relaxing with increasing swelling time. However with increasing number of FTC, the gel acquires increasing crystallinity which restricts the mobility of PVA and starch chains and consequently results in a suppressed swelling ratio. Peppas and co-workers (28) also noticed that the swelling ratio of cryogel decreases with increasing number of FTC due to an increased in crystallinity and crosslinking density within the hydrogel. An increase in mechanical strength of gel has also been noticed by some workers (29).

3.4.3. *Effect of pH*

In the present investigation the influence of pH on the swelling ratio of the cryogel has been studied by varying pH the swelling medium in the range 1.8 to 10.0. The results are shown in Figure 6 which clearly reveal that the swelling ratio of cryogel constantly decreases in the whole studied range. The decrease observed in the swelling ratio of the cryogel with increasing pH may be explained by the fact that although both the PVA and starch are non-ionic polymers, however, when pH of the swelling bath increases, the ionic concentration of external medium increases. The increased ionic concentration of swelling bath results in a

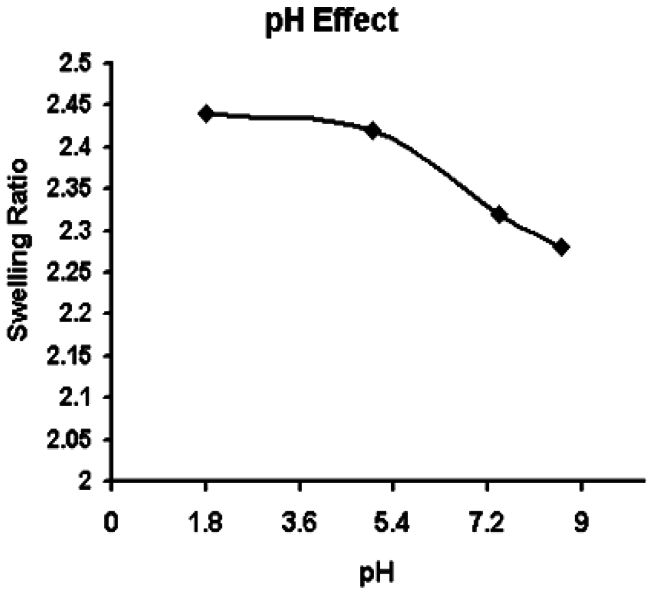

Fig. 6. Effect of pH on Starch and PVA blend.

fall in the ion osmotic pressure of the cryogel (30) which translates into lower swelling ratio of the blend.

3.4.4. *Effect of Temperature*

The influence of temperature on the swelling of the hydrogel is of great significance because it directly controls the diffusion of water molecules into the gel segmental mobility of the network chains and water-polymer interaction.

In the present study the effect of temperature on the degree of water sorption has been investigated by carrying out water sorption experiments in the range 15°C to 40°C. The results are presented in Figure 7. Swelling ratio constantly increases with increasing temperature of the swelling medium. The results may be explained by the fact that when the temperature is increased both the segmental mobility of cryogel chains and diffusion of water molecules into the gel increase which obviously results in a greater swelling.

3.4.5. *Effect of Biological Fluids*

It is well established that the equilibrium swelling behavior of a polymer network in solvent is the result of a balance between osmotic and restoring elastic pressure. The presence of salts in the surrounding aqueous medium is capable of tilting this balance which may result in either in an increase or decrease in swelling. In the present study, the effect of biological fluids has been examined by performing swelling experiments in the presence of urea, D-glucose (5%w/v), potassium iodide (KI) (15%w/v) and the physiological fluids such as saline water (0.9%NaCl) and artificial urine. The results are summarized in Figure 8, which clearly show that the presence of solute suppress the swelling ratio due to a decrease in osmotic pressure of the external solution.

3.5 Evaluation of Biocompatibility

The selection of a material to be employed as a biomaterial for a specific end use must meet several criteria such as physicochemical properties, function desired, nature of the physiological environment, adverse effects in the case of failure, expected durability and consideration relating to cost and case of production. Whatever the type of materials, the biocompatibility is the foremost requirement for all biomaterials. In the present study, the assessment of biocompatibility has been made on the basis of three *in vitro* tests viz. BSA (bovine serum albumin) adsorption

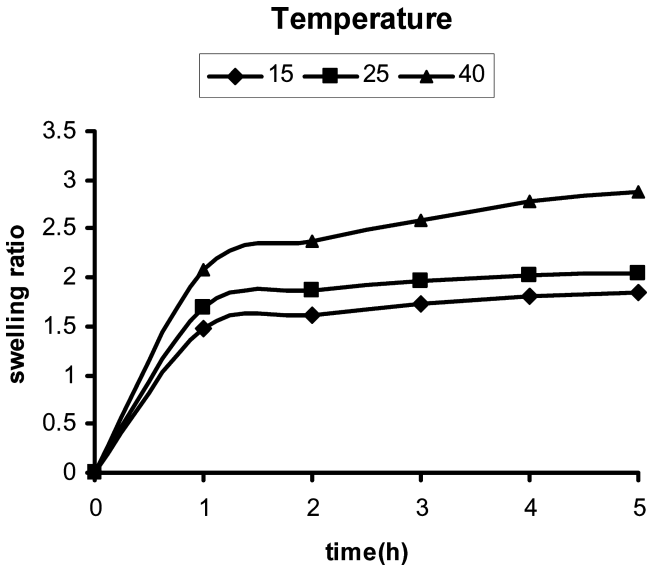

Fig. 7. Effect of temperature on Starch and PVA blends.

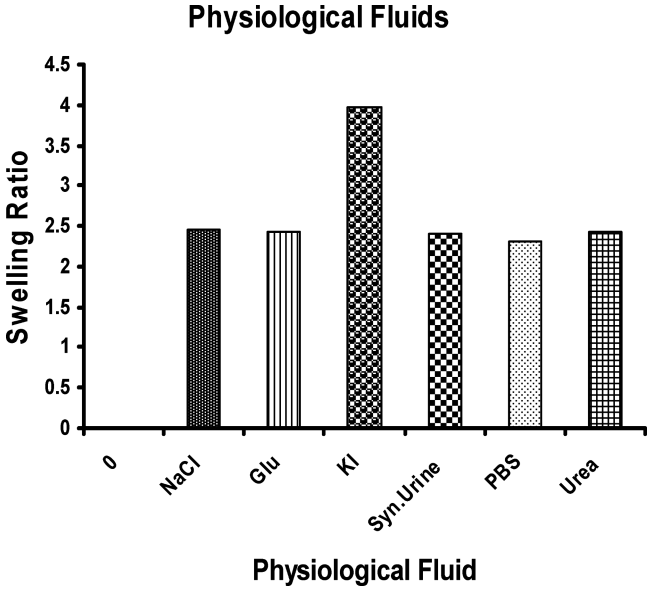

Fig. 8. Effect of Biological fluids on Starch and PVA blends.

Table 1. Data showing the biocompatibility parameters with varying composition of cryogel

PVA/starch %(W/W)	*Freeze–thaw cycles*	*% Hemolysis*	*BSA adsorbed (mg g^{-1})*	*Blood Clot (mg)*
20/80	3	18.19	0.0092	0.0008
33.3/66.7	3	16.24	0.0075	0.0006
42.8/57.2	3	13.28	0.0022	0.0003

test, blood-clot formation and hemolysis assay as discussed below:

(1) BSA adsorption

In the present work, the *in vitro* biocompatibility of prepared blends has been judged by monitoring the amount of protein (BSA) adsorbed by the blend. The results are shown in Table 1, which indicates that the amount of adsorbed BSA decreases with increasing wt. fraction of PVA in the range 20 to 44 (%w/w). The observed findings may be explained on the basis of the fact that PVA is non ionic, biocompatible and hydrophilic polymer and it does not provoke either any damage of blood cells or any change in the surface of plasma proteins which are the main factors for the biocompatibility.

The data summarized in the Table also indicates that as the wt. fraction of starch increases in the range 56 to 80 (%w/w), the amount of adsorbed BSA increases which suggests increasing thrombogenicity. The increase observed with increasing starch content may be explained by the fact that with increasing starch content, the wt. fraction of PVA decreases which results in a less biocompatible nature of the matrix. Moreover, increasing starch content may bring about heterogeneity in the blend matrix which may activate adsorption of the protein.

From the Table, it is also clear that a marginal increase in adsorbed BSA with increasing number of freeze-thaw cycles is due to the fact as the number of freeze-thaw cycles increases the swelling ratio decreases, i.e., gel acquires a lower degree of hydrophilicity and results in higher adsorption of BSA protein on the surfaces.

(2) Blood clot formation

The blood clot experiments were performed on different compositions of the hydrogels and the amounts of blood clot formed on the surfaces were recorded. The results are summarized in Table 1, which indicate that a much less amount of blood clot is formed. This obviously implies an antithrombogenic nature of the hydrogel surfaces.

(3) Haemolysis test

The prepared blends were also tested for hemolytic activity and the results obtained are shown in Table 1. The results obtained clearly indicate that with increasing PVA content, the extent of haemolysis constantly decreases while with a blend prepared by greater number of freeze-thaw cycles, the percent haemolysis increases. The observed results are attributed to the reason that with increasing wt. fractions of PVA in the blends, the surface composition favourably changes which improves the blood compatible quality of the material. On the other hand, with an increase in the number of FTC, the gel becomes more compact and heterogeneous and provides a greater chance for interaction between the blood components and gel pores. This obviously causes an increase in % haemolysis which implies enhanced thrombogenicity.

3.6 % Porosity

Porosity characterization is based on the presence of open pores, which are related to properties such as permeability, and surface area of the porous structure. The percent porosity data of different starch –PVA blends are summarized in Table 2, which clearly shows that the extent of porosity is significantly dependant on the chemical composition of the blend.

The results clearly reveal that when the weight fraction of PVA varies in the range 20 to 44% w/w, the porosity significantly increases. The observed increase in porosity may be attributed to the reason that with increasing wt. fraction of PVA in the blend, the hydrophilicity also increases due to hydrophilic nature of PVA. Due to enhanced hydrophilicity greater number of water molecules are attached to the macromolecular chains and, therefore, upon carrying out freezing-thawing cycles the ice crystal of larger dimension are formed which during thawing porous results in formation of large pores (31, 32).

3.7 Macroporous Nature

The freeze-thaw technique is an important tool in designing macroporous architectures with controlled porosity so that they may be desirably applied for specific tissue engineering applications. In the present study, various porous structures of PVA-starch blends have been fabricated by varying the number of freeze-thaw cycles so that the blends surfaces with different morphologies are produced. To

Table 2. Data showing the percent porosity with varying composition of cryogel

PVA/starch %(W/W)	*Freeze–thaw cycles*	*%Porosity*
20/80	3	22.3
33.3/66.7	3	48.22
42.8/57.2	3	81.9

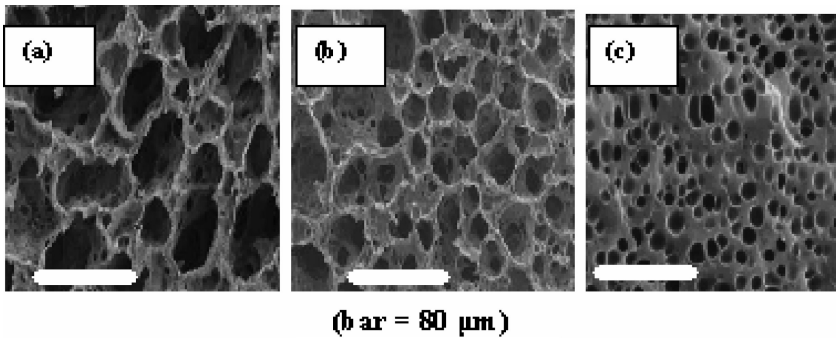

Fig. 9. Morphology of various cryogels prepared with varying FTC, (a) 3, (b) 5, and (c) 7.

achieve this, the blend solution of PVA and starch was subjected to three, five, seven and nine freeze thaw cycles and cryogels produced were examined by SEM technique. The results are shown in Figure 9(a) to (c), which clearly shows that the pore sizes gradually decreases with increasing number of FTC as is evident from the SEM images. The reason for the observed changes in morphologies of the gels may be attributed to the fact that with increasing FTC, the gel becomes more crosslinked and compact which eventually results in the reduction of pore sizes of the gels.

4 Conclusions

Repeated freezing and thawing of PVA and starch together produces highly elastic, water absorbing and blood compatible spongy cryogels.

It is noticed that the water sorption property of cryogel is greatly determined by the chemical composition of the network. When the concentration of PVA and starch increase in the gel, the swelling ratio of the cryogel constantly increases and beyond a definite concentration of PVA it starts decreasing. While in the case of increasing starch concentration, the degree of swelling initially increasing and beyond a definite concentration, it gradually falls.

The increasing number of freezing-thawing cycle (FTC) also results in a constant fall in the amount of water sorption.

The extent of water sorption by the cryogel is found to increase from acidic to natural pH range, while a fall in the swelling ratio is noticed with increasing pH in the alkaline pH range. The swelling ratio increases with increasing temperature of the swelling medium while it decreases when the concentration of electrolyte (NaCl) is increased in the outer aqueous medium. A lower degree of swelling is also observed in simulated biological fluids like saline water, artificial urine, KI solution and D-glucose solution.

With increasing concentrations of PVA and starch, the biocompatibility of cryogel increases while a reduced biocompatibility is noticed with increasing number of freeze-thaw cycles. The cryogels were found to show significantly porous in nature which varied with a varying degree of freeze-thaw cycles as evident from the SEM images.

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