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Designing of macroporous biocompatible cryogels of PVA–haemoglobin and their water sorption study

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Abstract Macroporous polymeric materials are three-dimensional porous architectures having enormous utility in the areas of biomedical, biotechnological and separation sciences. Thus realizing the crucial role of macroporous polymeric materials in tissue engineering and allied fields the present paper discusses synthesis, characterization, and blood compatibility study of macroporous cryogels of PVA and haemoglobin. Biocompatible spongy and porous hydrogels of polyvinyl alcohol–haemoglobin have been synthesized by repeated freezing–thawing method and characterized by Infrared (FTIR), and ESEM techniques. The FTIR analysis of prepared cryogels indicated that haemoglobin was introduced into the cryogel possibly via hydrogen bonds formed amongst hydroxyl groups and amino groups present in PVA and haemoglobin, respectively. 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 effect of drying temperature on its water sorption capacity was also studied. The biocompatibility of the prepared cryogels was judged by in vitro methods of blood-clot formation, percent haemolysis and protein (BSA) adsorption.

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1 Introduction

Hydrogels form a specific class of polymeric biomaterials and they are defined as two or multicomponent systems consisting of a three dimensional networks of polymer chains and water filled within the space between the macromolecular chains. Depending on the properties of the polymer (polymers) used, as well as on the nature and density of the network joints such structures in equilibrium may contain enormous amounts of water. In the swollen state the mass fraction of water in a hydrogel is much higher than the mass fraction of polymer [1].

There are many applications in which a material is needed to swell rapidly in a fluid. Materials that imbibe large volume of water are very versatile in daily use and also have specialized applications. For example materials such as diapers and sanitary napkins must rapidly imbible large volume of biological fluids and retain strength without losing fluids to surrounding dress [2]. Superporous hydrogels (SPHs) are a new generation of hydrogels with pore size in the range of 100 μm or larger whereas the mesh size of a conventional hydrogel remains below 100 nm [3, 4]. The swelling kinetics of SPHs is much faster than that of conventional hydrogels and it usually takes few minutes against the conventional hydrogels to swell to equilibrium in several days. Rapid swelling of SPHs is due to interconnected pore networks that are formed while the matrix is being formed [5].

Among porous materials of various chemical and compositional types the macroporous materials owe a prime position in the area of materials science due to ever seen advancements in design and processing of these materials driven by the rapid growth of emerging applications like energy conversion and storage, environment friendly catalysis, sensors, tissue engineering, DNA sequencing, drug delivery, cell markers and photonics [6]. Since

homopolymers alone cannot meet such divergent demands in terms of both properties and performance, a composite or interpenetrating polymer network (IPN) of two or three different polymers would be a better approach [7].

Although synthetic polymers have been largely employed in preparation of cryogels [8, 9], however, use of biological polymers such as haemoglobin in hydrogel synthesis has attracted much attention in recent past. Polymeric biomaterials in recent years have shown increasing potential in biomedical applications. Recently a new type of supermacroprous cryogel materials have been developed which show great promise in various biological applications. Cryogels typically have interconnected macropores (or supermacropores) allowing unhindered diffusion of solutes of practically any size, as well as mass transport of nano and even microparticles. The unique structure of cryogels, in combination with their osmotic, chemical and mechanical stability, makes them attractive matrices for chromatography of biological particles/cells and for tissue engineering applications. The sponge-type gels with inter-connected pores in the range of 10–100 μm are synthesized under cryostatic conditions from agarose, gelatin, acrylamide, polyvinyl alcohol or any other suitable polymeric materials [10]. Patton and Palmer [11] prepared haemoglobin-poly(acrylamide) hydrogel and examined the physical properties of bovine haemoglobin (BHb) chemically cross-linked to a pH responsive polymer poly(acrylamide) with the good of taking advantage of the polymer's pH sensitivity to generate low-P_{50} oxygen carriers for application in physiological conditions. Patton and Palmer [12] synthesized lipogel particles encapsulating bovine haemoglobin vis photopolymerization of poly (*n*-isopropylacrylamide) (PNIPA) and poly (acrylamide) (PAAm) monomers within liposomal reactors for use as an artificial blood substitute. Uyasal et al. [13] synthesized haemoglobin imprinted hydrogels by using TBA, AAm, and IA monomers at different pHs and evaluated their adsorption capacity. Ribinovic et al. [14] prepared liposomal encapsulated haemoglobin particles as oxygen carrying fluids. A new generation of drug delivery system based on heparin coated poly (alkylcyanoacrylate) nanoparticles coupled to hemoglobin has been developed by Chauvierre [15] and examined as oxygen carriers. Takagi and Hayashi [16] prepared artificial or natural oxygen carrier by per fluoro carbon emulsion, haemoglobin and erythrocyte and estimated the nature of O_2 supply from O_2 binding oxygen carriers.

Hydrogels based on blends of PVA and biological macromolecules are known as 'bioartificial polymeric materials [17] and have been extensively evaluated as potential matrices for the release of human growth hormone and other macromolecular drugs [18].

Thus realizing the biological and biomedical significance of haemoglobin the authors were motivated to prepare and characterize blend hydrogels of PVA and haemoglobin by repeated freezing–thawing cycles and subsequently to study the water sorption and blood compatibility of these cryogels. Easy preparation of the cryogels, their high swelling and fair blood compatibility make this approach attractive and broadly applicable in biotechnology and biomedical fields.

2 Experimental

2.1 Materials

Poly(vinyl alcohol) (PVA) (degree of hydrolysis 98.8% mol. wt. Ca 70000 Da) was purchased from Merck, India and used without any pretreatment. Haemoglobin (MW 67000, Isoelectric point 6.8) was supplied by Merck, India and used as received. All the other chemicals were of analytical reagent grade and triple distilled water was used in preparation of all solutions.

3 Methods

3.1 Preparation of cryogel

The freeze–thaw method was adopted in preparing blend hydrogels of PVA and haemoglobin as reported elsewhere [19]. In a typical experiment, 1.0 g of haemoglobin and 1.0 g of PVA were dissolved in 25 ml of 1 M NaOH solution in a petridish (diameter 4", Corning) and kept at −20°C for 24 h. The frozen gel was then thawed for 2 h at room temperature (25°C) and again kept at −20°C for freezing. Such freezing–thawing cycles were repeated at least thrice so that whole mass converted into soft, spongy, brown-black coloured hydrogels. The gel so prepared was purified by equilibrating it in distilled water for 72 h so that all unreacted chemicals were leached out. The swollen gel was cut into smaller discs and dried at room temperature for a week. The dried cryogel pieces (buttons) were stored in air-tight polythene bags.

3.2 Purification of gel

The gels as prepared above were further purified by equilibrating them in distilled water for 72 h. During the swelling process, the unreacted polymers, i.e. PVA and haemoglobin were leached out into the swelling bath and their concentration were determined spectrophotometrically by measuring the absorbance at 690 nm [20] and 420 nm [21], respectively (Systronic UV-visible spectrophotometers, Model No. 2201, Ahemdabad, India). The amount of PVA and haemoglobin leached out after gel formation are presented in Table 1.

Table 1 Data showing the composition of the feed mixture and prepared cryogels

Feed composition		Amount leached out		Real composition	
PVA (g)	Hb (g)	PVA (g)	Hb (g)	PVA (g)	Hb (g)
0.5	1.0	0.08	0.14	0.42	0.86
1.0	1.0	0.21	0.18	0.79	0.82
1.5	1.0	0.35	0.19	1.15	0.81
2.0	1.0	0.39	0.21	1.61	0.79
1.0	0.5	0.04	0.07	0.96	0.43
1.0	1.0	0.05	0.18	0.95	0.82
1.0	1.5	0.05	0.25	0.95	1.25
1.0	2.0	0.08	0.36	0.92	1.64

Hb Haemoglobin

3.3 FTIR spectral analysis

The infrared spectral analysis of the prepared hydrogels were performed on an FTIR spectrophotometer (Perkin Elmer, 1000 Paragon) by recording the IR spectra of the sample in the range 4000–500 cm^{-1}.

3.4 ESEM studies

The morphological features of the cryogels were investigated by recording their Scanning Electron Micrographs (STEREO SCAN, 430, Leica, SEM, USA) at 600X magnification, 20 kV voltage and 0.98 torr spot pressure.

3.5 Water uptake measurements

A conventional gravimetric procedure [22] was followed for monitoring progress of water uptake process. In brief, a pre-weighed dry piece of cryogel was immersed into distilled water at a definite temperature, taken out at predetermined time intervals, gently pressed between two filter papers to remove excess water and finally weighed by a sensitive balance (APX-203, Denver, Germany). The degree of water sorption was quantified in terms of the swelling ratio as calculated below:

$$\text{Swelling Ratio} = \frac{\text{Weight of the swollen gel } (W_s)}{\text{Weight of the dry gel } (W_d)} \quad (1)$$

The kinetic data were analyzed in the light of the following equations [23],

$$\frac{W_t}{W_\infty} = kt^n \quad (2)$$

and,

$$\frac{W_t}{W_\infty} = 4\left(\frac{Dt}{\pi L^2}\right)^{0.5} \quad (3)$$

where, W_t and W_∞ are the water intakes at time t and equilibrium time, respectively, L is the thickness of dry spongy gel, k is the swelling rate front factor, and n is the swelling exponent. When $n = 0.5$, the swelling process is of Fickian nature and is diffusional controlled while the value of n between 0.5 and 1.0 suggests for non-Fickian diffusion or more specifically anomalous diffusion. When n becomes exactly equal to unity, then the diffusion is termed as Case II. In some cases, the value of n has been found to exceed unity and it has been termed as super case II transport. In Eq. 3, D is diffusion coefficient of water molecules and L being the thickness of the dry gel. The value of n and D for different blend compositions were calculated and summarized in Table 2.

3.6 Measurement of solvent penetration velocity

The penetration velocity for each cryogel composition was determined by weight gain method as described by Peppas and Franson [24]. The penetration velocity was calculated from the slope of the initial portion of the penetration uptake curve from the equation given below:

$$V = \left[\frac{dW_g}{dt}\right] \cdot \left[\frac{1}{\rho}\right] \cdot \left[\frac{1}{2A}\right] \quad (4)$$

where V denotes the penetration velocity, dW_g/dt denotes the slope of the weight gain vs time curve, ρ is the density of the swelling solvent, A is the area of one face of disc and 2 accounts for the fact that penetration takes place through

Table 2 Data showing the kinetic parameters for the swelling process of PVA–haemoglobin cryogels

PVA (g)	Haemoglobin (g)	n^a	Diffusion constant $D \times 10^6$ ($cm^2\ s^{-1}$)[a]	Penetration velocity $V \times 10^3$ ($cm\ s^{-1}$)[a]
0.42	0.86	0.13 ± 0.03	2.8 ± 0.09	6.388 ± 0.88
0.79	0.82	0.68 ± 0.07	11.1 ± 0.99	8.597 ± 0.90
1.15	0.81	0.57 ± 0.06	20.8 ± 1.68	7.985 ± 0.75
1.61	0.79	0.63 ± 0.07	20.4 ± 1.55	7.985 ± 0.78
0.96	0.43	0.50 ± 0.06	9.1 ± 0.83	5.323 ± 0.40
0.95	0.82	0.68 ± 0.09	11.1 ± 0.99	8.597 ± 0.90
0.95	1.25	0.55 ± 0.04	16.1 ± 0.90	6.654 ± 0.83
0.92	1.64	0.57 ± 0.03	9.7 ± 0.80	3.993 ± 0.38

[a] The values represent meaning ± standard deviation of three determinations

Table 3 Data showing the biocompatibility parameters with varying composition of Cryogels

PVA (g)	Haemoglobin (g)	Freeze–thaw cycles	Blood clot (mg)	% Hemolysis	BSA adsorbed (mg g^{-1})
0.42	0.86	3	18.2	45.36	40.11
0.79	0.82	3	14.5	23.14	20.51
1.15	0.81	3	13.1	22.59	16.21
1.61	0.79	3	10.3	22.10	16.10
0.96	0.43	3	20.5	30.10	25.12
0.95	0.82	3	14.5	23.14	20.50
0.95	1.25	3	11.2	20.15	20.11
0.92	1.64	3	11.1	19.10	19.12
0.78	0.80	5	14.9	24.75	21.12
0.86	0.82	7	15.7	25.86	21.86
0.83	0.79	9	16.3	26.32	22.09
Glass surface			30.0	–	–
PVC (Blood Bag)			1.0	–	–

both faces. The penetration velocities calculated for different cryogel compositions are listed in Table 2.

3.7 Effect of pH

The effect of pH on the swelling ratio of the cryogels has been investigated by varying pH of the swelling medium in the range 2.0–9.2 by addition of 0.1 N HCl and 0.1 N NaOH. The pH measurement were recorded on a pH meter (Systronic, Ahemdabad, India).

3.8 Effect of temperature

The effect of temperature on the swelling ratio of the cryogels has been studied by varying the temperature of the swelling medium in the range 5–25°C.

3.9 Protein interaction and haemocompatibility study

The following test procedures have been adopted to judge the in vitro blood compatibility of the prepared cryogels:

3.9.1 Bovine serum albumin (BSA) adsorption

Adsorption of BSA onto the hydrogels was performed by the batch process as reported in other communications [25]. For adsorption experiments, protein (BSA) solutions were made in 0.5 M PBS (phosphate buffer saline) at physiological pH 7.4. A fresh solution of BSA was always prepared for every adsorption experiment. Prior to adsorption experiments, the hydrogel buttons were equilibrated with PBS for 24 h. The adsorption was then carried out by gently shaking a BSA solution of known concentration containing pre-weighed and fully swollen gels. By taking fully swollen gels, the possibility of soaking the BSA solution within the gel is minimized. Shaking was performed so gently that no froth was produced, otherwise it would have formed an air water-interface. After a definite time period, the gels were removed and the protein adsorption was assayed for the remaining concentration of BSA by recording the absorbance of protein solution at 272 nm on a UV spectrophotometer (Systronic, Model No. 2201, India). The adsorbed amount of BSA was calculated by following the weight balance equation,

$$\text{Adsorbed BSA} = \frac{(C_o - C_e)\ V}{m} \tag{5}$$

where C_o and C_e are the initial and equilibrium concentrations of BSA solution (mg ml^{-1}), V is the volume of protein solution and m is the mass of fully swollen gel, i.e., the adsorbent.

3.9.2 Clot formation test

Cryogels surfaces were examined for their blood compatibility by the blood-clot formation test as described elsewhere [26]. In brief, the cryogels were equilibrated with saline water (0.9% NaCl solution) for 1 day in a constant temperature bath. To these water swollen and equilibrated samples were added 0.5 ml of acid citrate dextrose (ACD) blood (from a healthy donor) and 0.03 ml of $CaCl_2$ solution (4 M) to start the thrombus formation. The reaction was stopped by adding 4.0 ml of deionized water and the clot formed was separated by soaking in water for 10 min at room temperature and then fixed in 30% formaldehyde solution (2.0 ml) for further 10 min. The fixed clot was placed in water for 10 min and after drying its weight was recorded. The same procedure was repeated for a poly(vinyl chloride) (PVC) film (negative control) (0.05 m × 0.05 m)

cut from a commercial blood-bag (Eastern Medicated Ltd., India) and glass (+ve control), respectively. The blood clot data are summarized in Table 3.

3.9.3 *Haemolysis assay*

Haemolysis assay experiments were performed on the surfaces of the prepared cryogels as described elsewhere [27]. In a typical experiment, cryogel films (4 cm^2) were 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 cryogel films. After 20 min, 2.0 ml of 0.9% sodium chloride (saline) was added to each sample to stop haemolysis and the samples were incubated for 60 min at 37°C. Positive and negative controls were obtained by adding 0.25 ml of human ACD blood and 0.9% NaCl solution, respectively to 2.0 ml of bidistilled water. Incubated samples were centrifuged centrifuge for 45 min, the supernatant was taken and its absorbance was recorded on a spectrophotometer (Systronic, Model No. 106, India) at 545 nm. The percentage haemolysis was calculated using the following relationship,

$$\% \text{ Haemolysis} = \frac{A_{\text{test sample}} - A_{(-)\text{ control}}}{A_{(+)\text{ control}} - A_{(-)\text{ control}}} \quad (6)$$

where A = absorbance, the values for the absorbance of the positive and negative control were 1.784 and 0.050, respectively.

3.10 Statistical analysis

All presented swelling results have been expressed as mean ± S.D. from three independent determinations. The value of n and D were obtained by plotting log (W_t/W_∞) vs. log t, and (W_t/W_∞) vs. $t^{1/2}$ curves, respectively, for each determination using least square method of best fitting and determining their respective slopes to yield the values of n and D.

4 Results and discussion

4.1 Mechanism of cryogel formation

In the present study, since a mixture of PVA and haemoglobin solution was used, all these three models hydrogen bonding, polymer crystallite, liquid–liquid phase separation may be considered to become operative simultaneously, thus, yielding a highly elastic gel. The reason for this supposition is that the haemoglobin is a functional biopolymer and its presence in PVA solution will enhance the process of hydrogen bond formation, polymer crystallite formation and phase separation.

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 crosslinking agents. Such physically crosslinked materials also exhibit higher mechanical strength than PVA gels crosslinked by chemical or irradiation techniques because the mechanical load can be distributed along the crystallites of the three dimensional structure [28].

The porous nature of cryogels produced by repeated freezing–thawing method may be explained due to the fact that whereas the freezing of a PVA–haemoglobin mixture results in the formation of ice crystal domains within the polymer mixture matrix, the thawing process results in melting of the ice crystal, thus, leaving wide pores in gel. A repeated performance of the two processes wides the pore sizes and thus, enhances the porous nature of the hydrogel.

4.2 FTIR spectra

The FTIR spectra of native PVA, native haemoglobin and PVA haemoglobin cryogel are depicted in Fig. 1a–c respectively which provides strong evidence for cryogel formation between PVA and haemoglobin. The spectra of the hydrogel shown as Fig. 1c clearly presents the strong characteristic peaks at 3450 and 1637 cm^{-1}, which are indicative of N–H stretching and C=O stretchings of

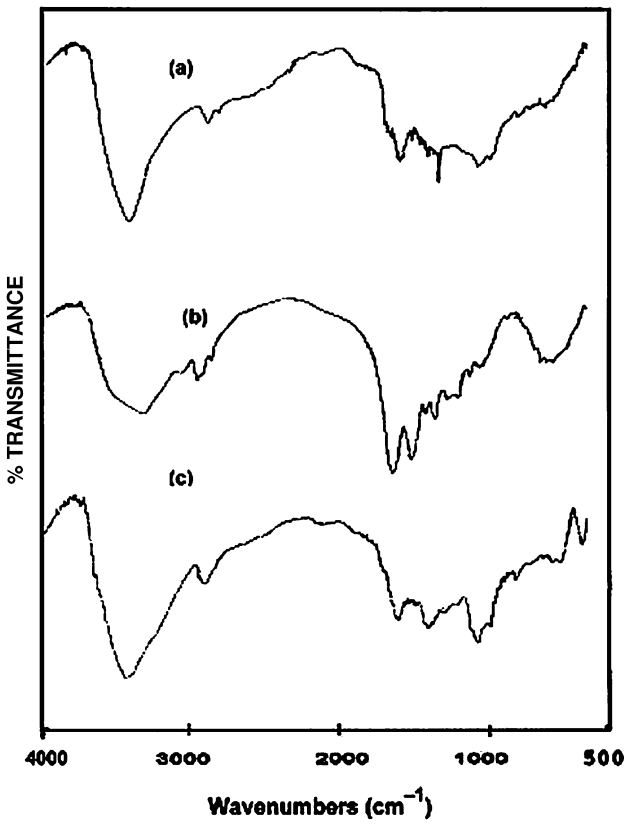

Fig. 1 FTIR spectra of (a) PVA, (b) Haemoglobin, (c) PVA–haemoglobin cryogel

haemoglobin, respectively. Spectra (c) clearly marks the combined presence of hydroxyl groups of PVA and amide groups of haemoglobin at 3450 cm^{-1}. This broad and strong peak appears due to the superimposition of N–H and O–H stretching which clearly indicates the formation of hydrogen bonding in the hydrogel. In addition to the above mentioned peaks, the IR spectra also confirms the presence of PVA and haemoglobin the cryogel as evident from the observed adsorption band at 3653.7 cm^{-1} (due to free –OH groups), 2935 cm^{-1} (due to C=O stretching, asymmetric N–H bonding due to NH_3^+), 1448 cm^{-1} (due to asymmetric bending of CH_3 groups and broad band due to NH_4^+ ion), 1331 cm^{-1} (due to interaction between O–H bending and C–O stretching), 1107 cm^{-1} (due to unconjugated C–N linkage), 624 cm^{-1} (broad peak due to hydrogen bonded out of plane –OH bending), respectively.

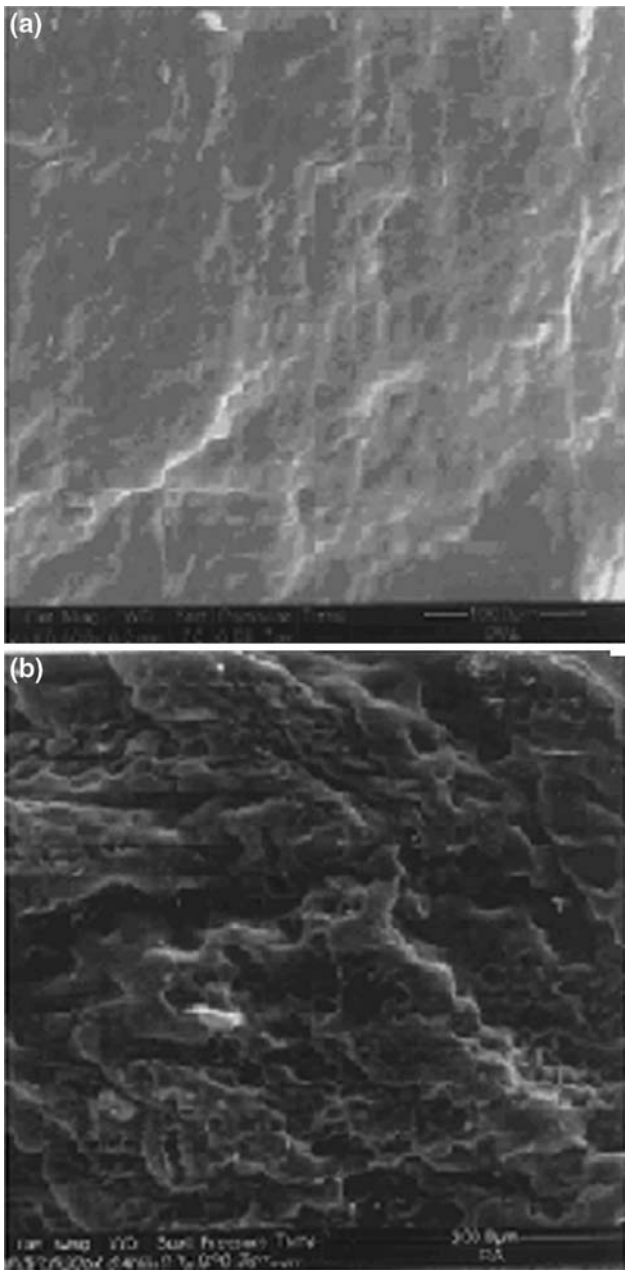

Fig. 2 Scanning electron micrographs (SEM) of **a** PVA and **b** PVA–haemoglobin

4.3 ESEM analysis

The ESEM images of both PVA and PVA–haemoglobin cryogels are shown in Fig. 2a, b, respectively. It is clear from the micrograph of Fig. 2a that PVA cryogel has homogeneous surface, the morphology of the cryogel significantly changes upon addition of haemoglobin as depicted in Fig. 2b. The image of Fig. 2b clearly reveals that macroporous domains are developed due to aggregation of PVA and haemoglobin molecules in the cryogel. The observed morphology, thus, supports the mechanism of pores formation in the cryogel.

4.4 Water sorption measurements

4.4.1 Effect of PVA

The influence of increasing concentration of PVA on the degree of water sorption was investigated by varying PVA in the range 0.42–1.61 g in the feed mixture of the cryogel. The results are shown in Fig. 3, which clearly indicate that the swelling ratio decreases with increasing concentration of PVA. The observed results may be explained by the fact that with an increase in PVA concentration, the volume fraction of polymer in the cryogel increases, which enhances the degree of interaction between the PVA–PVA

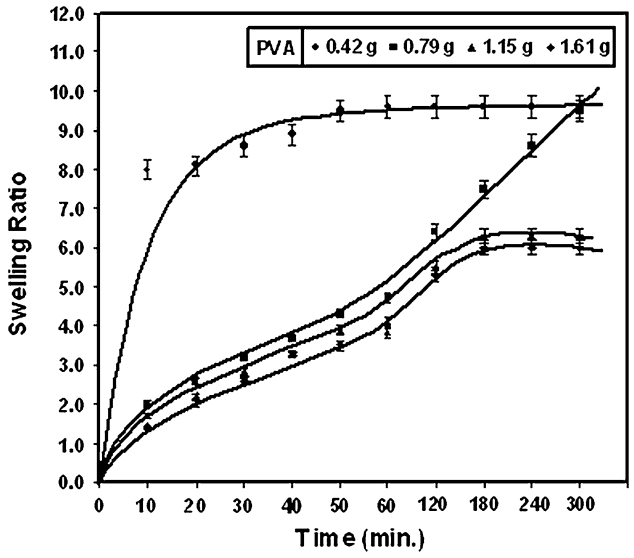

Fig. 3 Effect of varying amount of PVA on the swelling ratio of the cryogel of definite composition [Haemoglobin] = 0.82 g, Freeze–thaw cycle (FTC) = 3, Temp. = 25 ± 0.2°C

and PVA–haemoglobin molecules. Thus, an increased extent of crosslinking in the hydrogel results in a fall in the swelling ratio. The observed results may be explained by the fact that since PVA itself has a tendency to form reversible gel and the increasing amount of PVA results in a large number of crystallites formed.

4.4.2 *Effect of haemoglobin*

Inclusion of a hydrophilic polymer into a gel network is expected to enhance its water sorption capacity. In the present investigation, the effect of increasing haemoglobin concentration in the range 0.00–1.64 g in the feed mixture of the cryogel has been studied on the swelling characteristics of the hydrogel. The results are displayed in Fig. 4, which clearly reveal that the swelling ratio increases with increasing concentration of haemoglobin up to 0.82 g while beyond it, a fall is noticed. The observed results may be explained by the fact that since haemoglobin itself has a natural tendency to form reversible gel, its increasing concentration in the PVA–haemoglobin mixture lowers the concentration of PVA in the feed mixture and as a consequence upon successive freezing–thawing, the pore sizes of the cryogel network increases as confirmed by others also [13, 29, 30].

However, on further increasing the haemoglobin, i.e., beyond 0.82 g the decrease in swelling ratio, may be attributed to the fact that at higher concentration, haemoglobin, itself initiates formation of reversible gel which develops crystalline regions in the cryogel. This obviously results in low swelling ratio of the blend hydrogel.

4.4.3 *Effect of number of freezing–thawing cycle (FTC)*

In the present investigation, the effect of the number of freeze–thaw cycles (FTCs) on the swelling ratio of the cryogel was studied, as shown in Fig. 5. The results clearly indicate that the extent of swelling decreases with an increasing number of FTCs. The morphology of the swelling curves indicate that, whereas after the third cycle, the swelling ratio constantly increases up to 4 h and thereafter attain a limiting value, in the case of 5, 7 and 9 cycles the equilibrium swelling is attained much earlier and the time to attain equilibrium swelling decreases with an increasing number of FTCs. The observed results may be attributed to the fact that in case of 3rd cycle, the cryogel does not have large crystalline regions and therefore, the chains of PVA and haemoglobin 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 haemoglobin chains and, consequently, results in a suppressed value of swelling ratio. Peppas and co-workers [31] also noticed that the swelling ratio of cryogel decreases with increasing number of FTC due to an increase in crystallinity and crosslinking density within the hydrogel.

4.4.4 *Effect of pH*

pH responsive hydrogels constitute an important class of biomaterials, which play a significant role in designing

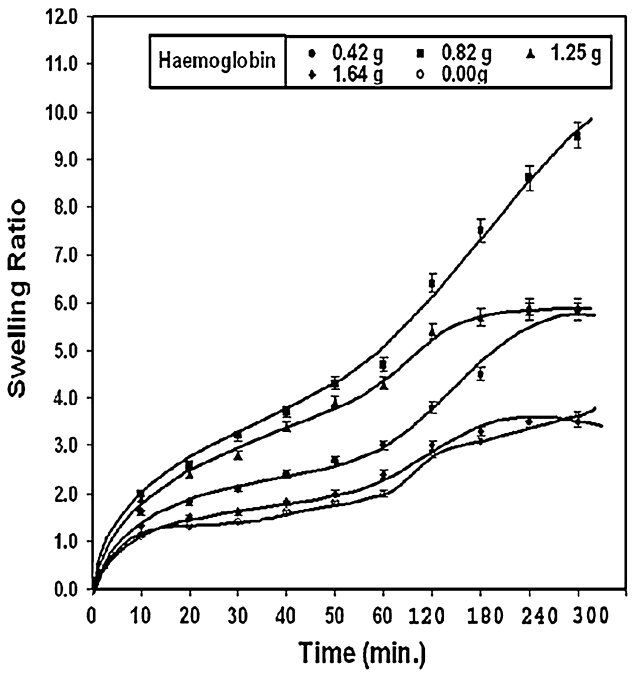

Fig. 4 Effect of varying amount of Haemoglobin on the swelling ratio of the cryogel of definite composition [PVA] = 0.79 g, [FTC] = 3, Temp. = 25 ± 0.2°C

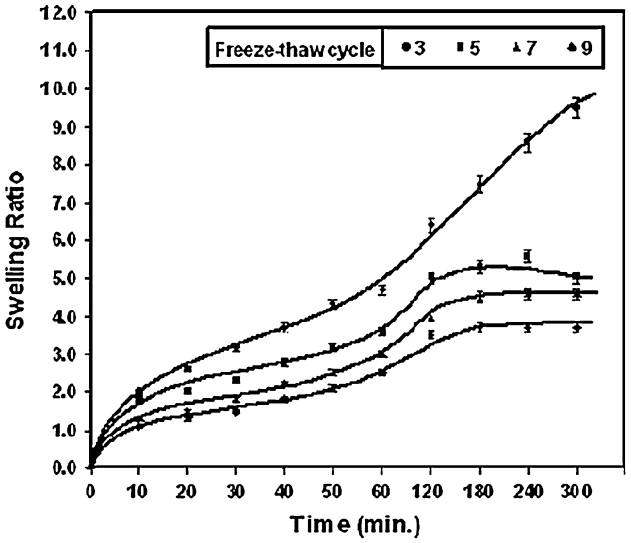

Fig. 5 Effect of number of Freeze–thaw cycle (FTC) on the swelling ratio of the cryogel of definite composition [PVA] = 0.79 g, [Haemoglobin] = 0.82 g, [FTC] = 3

targeted drug delivery [32]. The underlying principle for targeted drug delivery is the pH controlled swelling of hydrogel which normally results from the change in relaxation rate of network chains with changing pH of the swelling medium.

The effect of pH on the swelling ratio of the cryogel has been studied by varying the pH of the swelling medium in the range 2.0–9.2. The results are shown in Fig. 6 which clearly reveal that the swelling ratio of the cryogel increases up to pH 7 and achieve an optimum swelling and, thereafter, decreases with further increase in the pH.

The observed results may be attributed to the reason that in lower pH range, the haemoglobin molecules bear a net positive charge (due to $-NH_3^+$) and undissociated carboxyl groups (–COOH) which easily form hydrogen bonds with the hydroxyls (–OH) of the PVA, thus, resulting in the formation of crystallites in the gel. This obviously favours a lower swelling ratio of the cryogel in acidic medium. However, with increasing pH in acidic medium, the number of OH^- ions goes on increasing which after penetrating into the gel, produce repulsions, thus, causing relaxation of macromolecular chains of the gel network. This obviously facilitates diffusion of water molecules into the gel and, therefore, the swelling increases. However, beyond pH 7.0, i.e. in slight alkaline range, the network chains are so much relaxed that the hydrogen bonds established between the water molecules, the PVA and haemoglobin chains become weak and the entrapped water molecules are forced out, thus, resulting in a lower swelling ratio of the hydrogel. The expulsion of bound water molecules at large relaxation of macromolecular chains has also been observed elsewhere [33].

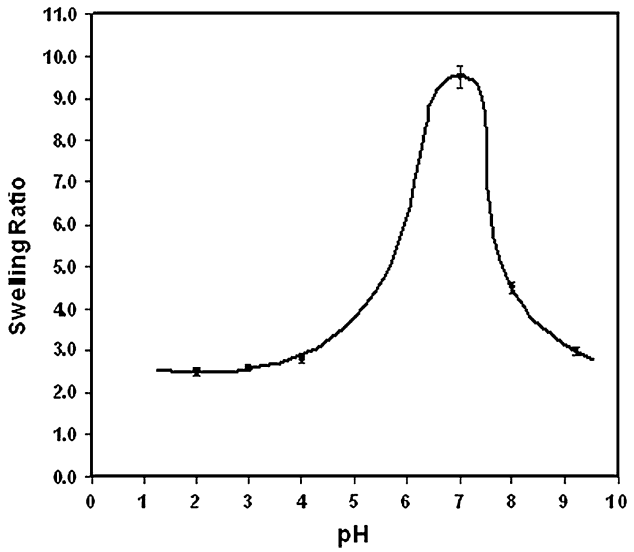

Fig. 6 Influence of pH on the swelling ratio of the cryogel of definite composition [PVA] = 0.79 g, [Haemoglobin] = 0.82 g, [FTC] = 3

4.4.5 *Effect of temperature*

In the present study, the effect of temperature on the swelling ratio of the cryogel has been investigated by varying the temperature of the swelling medium in the range 5–25°C. The results are depicted in Fig. 7, which reveal that the swelling ratio increases with increasing temperature of the swelling medium. The results can be explained by the fact that temperature has a direct influence on the swelling behaviour of a hydrogel as it affects both the segmental mobility of the hydrogel chains as well as the diffusion of penetrant molecules, therefore, with increasing temperature, the network chains also undergo faster relaxation due to increased kinetic energy and thus, facilitate the water sorption process. Also, both the segmental mobility of cryogel chains and diffusion of water molecules into the gel increase which results in a greater swelling:

In order to analyze the quantitative effect of temperature, the Gibbs–Helmholtz equation can be applied according to which [34],

$$\frac{d\ln(W_\infty)}{d(1/T)} = -\Delta H_m/R \qquad (7)$$

where R is a gas constant and ΔH_m is the enthalpy of mixing between the dry polymer and infinite amount of water. When $\ln W_\infty$ is plotted against reciprocal of temperature, a straight line with a negative slope is obtained (Fig. 8) which implies an endothermic process. The value of ΔH_m was calculated to be -28.90 kJ mol^{-1}, which clearly suggests for an endothermic nature of the swelling process.

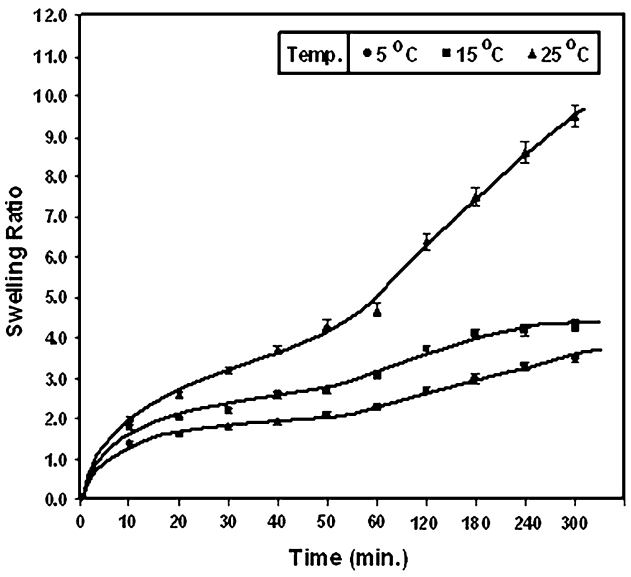

Fig. 7 Effect of temperature on the swelling ratio of the cryogel of definite composition [PVA] = 0.79 g, [Haemoglobin] = 0.82 g, [FTC] = 3

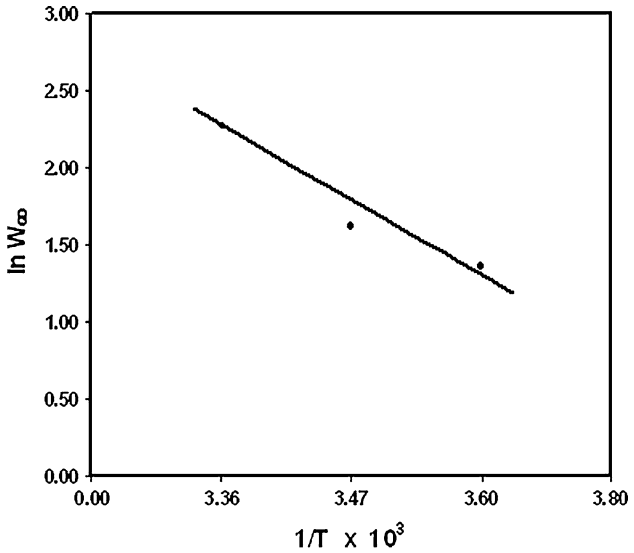

Fig. 8 Plot drawn between ln W_∞ and 1/T for the cryogel of definite composition [PVA] = 0.79 g, [Haemoglobin] = 0.82 g, [FTC] = 3

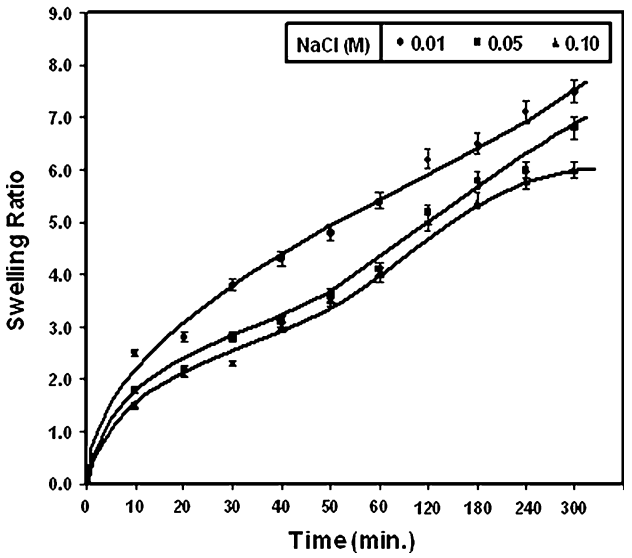

Fig. 9 Effect of NaCl on the swelling ratio of the cryogel of definite composition [PVA] = 0.79 g, [Haemoglobin] = 0.82 g, Temp. = 25 ± 0.2°C, [FTC] = 3

4.4.6 *Effect of salts*

The influence of the presence of salts in the swelling medium of a hydrogel is of importance in agriculture and biomedical applications, for example, in water reservoirs in agriculture, and for hydrogels as implants [35] for drug release applications. In principle, change in the swelling behaviour due to presence of salts can affect the mechanical properties of the material as well as the 'tortuosity' of the matrix which gives rise to different diffusion coefficients of drug release [36].

The effect of salts on the swelling ratio of the cryogel was studied by adding NaCl in the concentration range 0.01–0.1 M. The results are presented in Fig. 9, which clearly reveal that the swelling ratio decreases with increasing salt concentration in the swelling medium. The results may be explained by the fact that with increasing salt concentration, the osmotic pressure (π_{ion}) decreases due to a decrease in the concentration difference between mobile ions in the exterior and interior of the swelling hydrogel and, consequently the swelling ratio decreases. These results are quite usual and have been published by other workers also [37].

The prediction of swelling of a hydrogel can be best made by the Donnan equilibrium theory, according to which, the osmotic pressure is mainly shared by π ions which is caused by the counter ion difference between the gel and the external solution. Thus, as proposed by Donnan,

$$\pi_{ion} = RT\,\Sigma(C_i^g - C_i^s) \quad (8)$$

where C_i is the mobile ion concentration of species i and superscripts g and s represent the gel and solution phase, respectively. With increasing salt ion concentration, osmotic pressure (π_{ion}) decreases due to a decrease in ($C_i^g - C_i^s$) term in equation and consequently, the swelling ratio decreases.

4.4.7 *Effect of biological fluids*

The effect of nature of the medium ion of biological fluids on the swelling ratio has been examined by performing swelling experiments in the presence of urea, D-glucose (5% w/v), potassium iodide (15% w/v), and in physiological fluids such as saline water (0.9% NaCl) and artificial urine. The results are depicted in Table 4, which clearly show that the presence of salt ions in the medium suppresses the swelling ratio due to a decrease in osmotic pressure of the external solution as equilibrium swelling behaviour of a polymer network in a solvent is the result of a balance between the osmotic pressure and the restoring elastic pressures.

Table 4 Data showing the swelling ratio of the cryogel in various simulated physiological media

Physiological fluids	Swelling ratio (after 5 h)[b]
Water	9.6 ± 0.41
KI (15% w/v)	4.9 ± 0.39
Urea (5% w/v)	5.8 ± 0.40
D-glucose (5% w/v)	5.9 ± 0.38
Saline water (0.9% w/v NaCl)	7.8 ± 0.49
Synthetic urine*	6.6 ± 0.31

* NaCl (0.8% w/v), $MgSO_4$ (0.10% w/v), urea (21% w/v) and $CaCl_2$ (0.06% w/v)

[b] The value represent mean ± standard deviation of three determinations

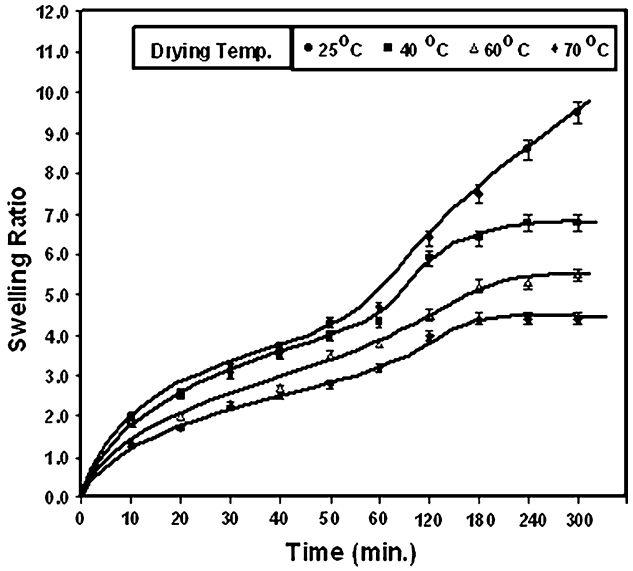

Fig. 10 Effect of drying temperature of the cryogel on the water-sorption capacity of the cryogel of definite composition [PVA] = 0.79 g, [Haemoglobin] = 0.82 g, [FTC] = 3

4.4.8 Effect of drying temperature on swelling

In the present case, the influence of drying temperature of the cryogel on its swelling ratio was investigated by drying the cryogels in the temperature range 25–70°C. The results are shown in Fig. 10, which clearly indicate that the swelling ratio drastically decreases with increasing drying temperature. The observed results may be attributed to the fact that, when the drying temperature is high, some of the crosslinks may be disrupted and this obviously allow freedom to both PVA and haemoglobin chains to form crystalline phases. Thus, an enhanced degree of crystallinity results in lower water sorption due to the reason that in crystalline polymers like cellulose [38], PVA [39], etc., swelling occurs mainly through the amorphous region.

4.5 Kinetic analysis of sorption data

Numerous factors, such as equilibrium water content, swelling rate and chemical composition of the system, contributes to the water transport mechanism in a swelling hydrogel. In the present study, the dynamic water sorption experiments were performed and data were analyzed in terms of swelling exponent n and diffusion constant D as summarized in Table 2.

It is clear from Table 2 that when the amount of PVA is varied in the range 0.42–1.61 g in the feed mixture of cryogel, the value of swelling exponent n increases from 0.13 to 0.63 in the non-Fickian region. This clearly indicates a shift of water transport mechanism from Fickian to anomalous type, i.e. from a diffusional controlled to relaxation controlled process. The observed results are due to their compact arrangement which results in a decreasing relaxation rates of network chains.

The variation in the haemoglobin content of the cryogel, in the range 0.00–1.64 g of haemoglobin content does not significantly affect the value of n which in the present case varies from 0.50 to 0.57, thus, suggesting a shift of water transport mechanism from Fickian to anomalous type, i.e. from a diffusion controlled to relaxation controlled process. The observed shift is due to the reason that number of haemoglobin molecules and their compact arrangement in the cryogel results in a decreasing relaxation rate of the network chains.

4.6 Evaluation of biocompatibility

Biocompatibility of a material demands several qualities to qualify as biomaterial. In the first place, it must be biochemically compatible, non-toxic, non-irritable, non-allergenic and non-carcinogenic; second, it must be biomechanically compatible with surrounding tissue; third, a bio-adhesive contact must be possible between the material and living tissues.

In the present study, the assessment of biocompatibility has been made on the basis of three in vitro tests, namely protein adsorption, blood-clot formation and haemolysis assay as discussed below.

4.7 Bovine serum albumin (BSA) adsorption

A hydrogel can be recognized as a biomaterial only when, apart from showing a water uptake property, it exhibits anti-thrombogenic behaviour, i.e., less tendency to initiate blood clot formation [40].

In the present study, the biocompatibility of prepared cryogel has been judged by monitoring the amount of protein (BSA) adsorbed by cryogel. The results are summarized in Table 3, which indicate that the amount of adsorbed BSA decreases with increasing amount of PVA and haemoglobin in the feed mixture of the cryogel. In fact, during the contact of foreign material with flowing blood not only BSA but other proteins like fibrinogen also tend to adsorbe over the material surface. The adsorption behaviour of proteins is not as simple as it looks or described. In addition to the conformational and other related structural changes the replacement of previously adsorbed proteins by the other approaching protein is also a complicated phenomenon. (Vorman Effect). The rational behind carrying out adsorption test is that the larger is the amount of protein adsorbed, greater would be the platelet addition and consequently blood clot formation. The observed results may be explained on the basis of the facts that both the PVA and haemoglobin are hydrophilic polymers and do not provoke either any damage of blood cells or any change in

the surface of plasma proteins which are the main prerequisites for biocompatibility. However, adsorption of BSA increases with increasing number of freeze–thaw cycles which is due to the fact as the number of freeze–thaw cycles increases, the swelling ratio decreases, i.e. gel acquires lower degree of hydrophilicity and results in higher adsorption of BSA protein on surfaces.

4.8 Blood clot formation

For many biomedical applications, it is desirable to have a high anti-thrombogenic potential of the biomaterial implanted into the body for some specific purpose. In the present investigation, the antithrombogenic property of the hydrogel has been judged by monitoring the amount of blood clot formed by blood clot formation test. The results summarized in Table 3, which clearly indicate that whereas the amount of the blood clot constantly decreases with increasing amount of PVA and haemoglobin in the hydrogel, a marginal increase in blood clot is noticed with an increasing number of freeze–thaw cycles. The results may be explained on the basis of fact that both PVA and haemoglobin are hydrophilic polymers and, therefore, are not expected to provoke either any damage of blood cells or any change in the structure of plasma proteins and cryogel surface acquires more smoothness and the water sorption results also indicate that with increasing number of freeze–thaw cycles, the swelling ratio decreases, i.e., gel acquires lower degree of hydrophilicity and this clearly results in a decreased antithrombogenicity of the gel and, consequently, a marginal increase in blood clot is observed as number of freeze–thaw cycle increases.

4.9 Haemolysis test

In the present study, the cryogels were also tested for haemolytic activity and the results are summarized in Table 3, which clearly indicate that with increasing the amount of PVA and haemoglobin amount in feed mixture of cryogel, the haemolysis constantly decreases, while with cryogels prepared with a greater number of freeze–thaw cycles, the haemolysis increases. The observed results are due to the reason that with changing PVA and haemoglobin, the surface becomes more blood compatible due to hydrophilic nature of the both. The results are consistent with the clot-formation results.

5 Conclusions

The repeated freezing and thawing of PVA and haemoglobin solution produces a highly elastic, water absorbing and blood compatible spongy hydrogel. The water sorption property of the cryogel is greatly determined by the chemical composition of the network. When the amount of PVA increases in the hydrogel, the swelling ratio of the cryogel constantly decreases, while in the case of increasing amount of haemoglobin the degree of swelling initially increases and beyond a definite concentration, it gradually falls. The increasing number of freezing–thawing cycles (FTCs) also results in a steady fall in the amount of water sorption.

The extent of water sorption by cryogel is found to increase from acidic to neutral medium while a fall in the swelling ratio is noticed with increasing pH in the alkaline range.

The swelling ratio increases with increasing temperature of the swelling medium whereas it decreases with the increase in the concentration of electrolyte NaCl in outer aqueous medium. The cryogels also show a lower swelling when dried at increasing temperature and also immersed in biological fluids. It is also found that with varying PVA and haemoglobin, the mechanism of water transport changes from Fickian to non-Fickian.

The prepared cryogels exhibit a fair degree of blood compatibility as evident from consistent results of protein adsorption, blood clot formation and haemolysis test. Biocompatibility also increases with increase in amount of PVA and haemoglobin but decreases with increasing number of freeze–thaw cycles.

It is thus clear that the prepared hydrogel matrices in the present offer useful and significant water sorption properties, structural and functional morphological features and over all good blood compatibility which are essential prerequisites for any material to qualify as biomaterial for suitable pharmaceutical and biomedical applications.

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