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Biomedical evaluation of polyvinyl alcohol–gelatin esterified hydrogel for wound dressing

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Abstract The wound is a biosynthetic environment in which numerous cellular processes are interlinked in the process of repair. Modern dressings are designed to facilitate wound healing rather than just to cover it. Hydrogel dressing can protect injured skin and keep it appropriately moist to speed the healing process by absorbing exudates while maintaining the products of tissue repair, including growth factor and lysosomes, in contact with the wound. The design and development of novel membrane of hydrogels prepared by esterification of polyvinyl alcohol with gelatin were attempted. Contact angle of goat blood was determined. The hydrogel was characterized by hemolysis test and water vapor transmission rate. Diffusion coefficient of salicylic acid (SA) and gatifloxacin, a fourth generation fluoroquinolone, through the membrane was determined. Both the drugs were used as model drug. Methyl tetrazolium dye assay of the membrane was done using L929 fibroblast cell line and mice splenocytes to establish the biocompatibility of the membrane. The equilibrium goat blood-in-air contact angles of measured ester films ranged from 56 to 60°. The hydrogel was found to be hemocompatible and moisture retentive indicating its possible use in moist wound care. The diffusion coefficient of SA and gatifloxacin through the membrane was found to be 1.49×10^{-5} and 3.97×10^{-6} cm^2/s respectively. The membrane was found to be compatible with the L929 fibroblast cell line and mice splenocytes.

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Introduction

Hydrogels are one of the most promising types of three-dimensional crosslinked polymers being used for new material development. Hydrogels do not dissolve in water at a physiological temperature and pH but swell considerably when put in physiological fluids or aqueous medium. These materials have been attracting much attention in medical fields due to their hydrophilic nature. They are being used in the medical device industry for the development of contact lenses, artificial corneas, dressing as coating for sutures, catheters and electrode sensors. Such a wide range of uses require easy manipulation of physical properties of the hydrogels. Since biocompatibility apparently depends on water content, characterization of the amount of imbibed water in the swollen gel is essential [1–3]. The increasing importance of hydrogels in areas like pharmaceutical and food chemistry, medicine and biotechnology, has stimulated theoretical and experimental work on the several properties of hydrogels in aqueous solutions. Radical polymerization of low molar mass monomers in the presence of crosslinking agents results in the formation of chemically crosslinked gels. The hydrogel characteristics (e.g. swelling and strength) can be modulated by varying the amount of crosslinker. Moreover, addition of special monomers can lead to the formation of stimuli sensitive materials (e.g. pH-sensitive gels, temperature-sensitive gels, pressure-sensitive gels) [4–8]. Water-soluble polymers owe their solubility properties to the

presence of functional groups (e.g. OH, COOH, NH_2), which plays an important role in the formation of hydrogels and water holding capacity. Crosslinking reaction leads to the formation of covalent linkages between polymer chains by the reaction of functional groups with complementary reactivity, such as an amine, carboxylic acid or an isocyanate–OH/NH_2 reaction [9]. In recent years, considerable research has been done on the characterization and swelling behavior of hydrogels prepared by simultaneous free radical copolymerization and crosslinking in the presence of an initiator and a crosslinking agent.

Polyvinyl alcohol (PVA) blends have long been used with other natural polymers due to its ready film forming ability. The performance properties of PVA are influenced by molecular weight and the degree of hydrolysis. The molecular mass of PVA is ~160 kD [10, 17]. PVA is having planar zigzag structure like polyethylene [11]. All PVA grades are readily soluble in water. As a hydrophilic polymer, PVA exhibits excellent water retention properties. Conditions for dissolution are primarily governed by the degree of hydrolysis, but are also influenced by other factors such as molecular weight, particle size distribution and particle crystallinity [12]. Optimum solubility occurs at 87–89% hydrolysis. The partially hydrolyzed grades in this range exhibit a high degree of cold-water solubility. For total dissolution water temperatures of about 85 °C with a hold time of 30 min is required.

Gelatin is prepared by the thermal denaturation of collagen either with acidic or alkaline treatment. Gelatin contains a large number of glycine residues (almost 1 in 3). The other major amino acids being proline and 4-hydroxyproline residues. Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides. Solutions of gelatin undergo coil-helix transition followed by aggregation of the helices. Higher levels of pyrrolidines in the gelatin structure result in stronger gels [13]. Gelatin films containing greater triple-helix content swell less in water and have better mechanical properties [14]. Chemical crosslinking agents alter the gel properties of gelatin, for example, transglutaminase is used for crosslinking lysine to glutamine residues [15] while glutaraldehyde is used to crosslink lysine to lysine residues. Gelatin can be obtained from animal skins and bones and sometimes from fish scales.

Like PVA, gelatin also possesses film-forming property. Accordingly it is used for making hard and soft gelatin capsules. Toshihisa et al. prepared PVA–gelatin blended films and studied its properties [16]. Considering the film forming property of PVA–gelatin blend, an esterified product of PVA–gelatin appears to be a good candidate for artificial skin. Hence attempts were made to develop a hydrogel membrane of an ester form of PVA–gelatin. Attempts were also made to characterize the hydrogel.

Materials and methods

Materials

PVA (mol. wt. 125,000) was obtained from s.d. fine-chem. Limited, Mumbai, India. Gelatin (for bacteriology purposes) and salicylic acid (SA) were obtained from Loba-Chemie Indoaustranal Co., Mumbai, India. Hydrochloric acid 35% pure was obtained from Merck Limited, Mumbai, India. Double distilled water was used throughout the study. Gatifloxacin, a fourth generation fluoroquinolone, was obtained as a gift from Ranbaxy laboratories limited, Gurgaon, India.

Methyl tetrazolium (MTT) dye, Dubelcco's minimum essential medium (DMEM) media, dimethyl sulphoxide (DMSO), L929 fibroblast cell line and mice were procured as gift from Department of Biotechnology, Indian Institute of Technology, Kharagpur, India.

Preparation of hydrogel

The preparation of the hydrogel has been described elsewhere [17]. In short, two and a half grams of gelatin was dissolved in 50 mL of 10% aqueous solution of PVA. Concentrated hydrochloric acid (HCl, 0.05 mL) was added and the resulting dispersion was stirred at 70 °C for half an hour to carry out esterification reaction between PVA and gelatin. The thick dispersion so obtained was converted into a membrane by the conventional solution casting method. The membranes so obtained were washed thoroughly to wash off the hydrochloric acid. The FTIR spectra of the hydrogel revealed shifting of >C=O peak of gelatin from 1,685 to 1,756 cm^{-1} indicating esterification of gelatin with PVA. XRD of the hydrogel indicated that the crystallinity of the hydrogel were mainly due to gelatin rather than PVA.

Contact angle measurement

The goat blood-in-air contact angles of ester films were measured at room temperature using the sessile drop method [18] by a Ramé-Hart goniometer and imaging system (Ramé-Hart Inc., Mouttain Lake, NJ) within 10 s after water dropping. Five independent measurements at different sites were averaged.

Hemocompatibility test

In the present work the hemocompatibility tests were carried out broadly on the basis of ASTM standard [19]. The test is mainly aimed at finding the extent of hemolysis caused in the presence of the sample prepared. The hemolysis percentage is defined as

$$\% \text{ Hemolysis} = \left\{\left[OD_{test} - OD_{negative}\right] / \left[OD_{positive} - OD_{negative}\right]\right\} \times 100 \quad (1)$$

For this purpose, goat's blood was collected in a beaker containing sodium citrate in the proportion of 3.8 g of sodium citrate per 100 mL of blood to avoid coagulation. The citrated blood was then diluted with normal saline in the proportion of 8:10. For checking hemolysis, 0.2 mL of diluted blood was added to 0.5 mL of 0.01N hydrochloric acid (HCl), which was further diluted to 10 mL with normal saline and incubated at 37 °C for 60 min. The optical density (OD) of the incubated solution was measured in a spectrophotometer at 545 nm. Since HCl is known to cause large-scale rupture of red blood corpuscles (RBC), the OD count of the solution was taken as positive control and was designated as $OD_{positive}$. Similarly, for negative control, 0.2 mL of diluted blood was diluted to 10 mL with normal saline and was incubated at 37 °C for 60 min. The OD of the solution was measured in a spectrophotometer at 545 nm and the same was designated as $OD_{negative}$. The reason for adding normal saline solution for negative control test was that it is known to cause least RBC rupture. Having obtained the two standard ODs, the OD of the test material was obtained in similar lines. Sample (5 × 5 mm) was taken in a standard test tube containing normal saline and was incubated at 37 °C for 30 min for providing temperature equilibrium. Diluted blood (0.2 mL) was then added to the test tube, mixed gently and incubated for 60 min. OD of the sample was designated as OD_{test}. As per accepted norm, for a hemolysis percentage less than 5, the test material was considered highly hemocompatible and a value less than 10 as hemocompatible.

Water vapor transmission rate

Water vapor transmission rate (WVTR) through the membrane was measured using the upright wet cup method as described by ASTM E96 [20], with a reduced air gap and membrane area (D = 6.32 mm) according to Hu et al. [21].

Measurement of diffusion coefficient

A diaphragm cell shown in Fig. 1 was used to measure the diffusion coefficient. The cell consisted of two chambers separated by a film (0.2 mm thick) of the hydrogel. The hydrogel membrane was equilibrated in water for 2 h before the experimentation. The first chamber contained 10 mL of aqueous solution of SA (8 mg/10 mL) (donor). The other chamber consisted of 100 mL of filtered distilled water (receptor). Then the donor was lowered so that the hydrogel just touched the receptor fluid, kept under stirring. The system was placed in a constant-temperature water bath. A pipette was used to draw 0.1 mL solution from donor and 1.0 mL sample from receptor periodically. The withdrawn samples were replaced with equal volume of distilled water. The samples were analyzed by acidic ferric chloride solution to determine the concentration of SA in each chamber as a function of time. The diffusion coefficient, D, was calculated from these results. The experimentation was conducted at room-temperature (30 °C).

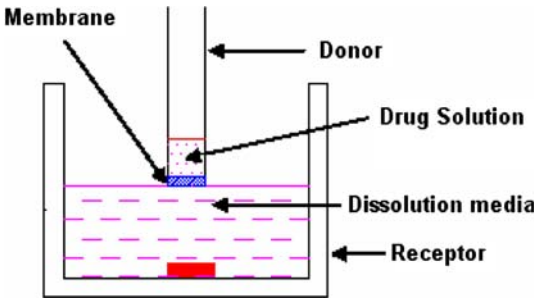


Fig. 1 Diagrammatic view of diffusion apparatus

In another experiment, 5 mL of aqueous solution of gatifloxacin (3 mg/mL) was taken in the donor. The receptor chamber contained 25 mL of ringer's solution. Ringer's solution was used in the receptor to simulate the wound conditions where there are a number of electrolytes. The rest of the procedure was similar to the above-mentioned one. Gatifloxacin concentration was determined by measuring absorbance at 291 nm in a spectrophotometer.

MTT Assay

L929 cell line

The hydrogel membrane was cut into 5 × 5 mm dimension and was transferred in the polystyrene petriplates. Samples were sterilized by pouring 70% ethanol in petriplates and keeping the same under UV-light in laminar hood until the alcohol evaporated. To the samples 20 μL of L929 cell suspension (1×10^6 cells/mL) was seeded and was kept in the incubator (37 °C) for 1 h to allow the cells to adhere to the matrix of the sample. After the cell adherence 2 mL of DMEM medium was added into each of the petriplates and was again incubated for 48 h for allowing cell proliferation. After 48 h of incubation 200 μL of MTT dye (4 mg/mL) was added and the system was again incubated for 3 h. After the incubation, the media from the petriplates were discarded and 400 μL of DMSO was added for the development of the color. For control the cells were seeded in petriplates. The color developed was measured spectrophotometrically at 570 nm.

Mice splenocytes

Mice were sacrificed and their spleens were removed aseptically. The cell suspension was prepared by means of loose potter and flushing. After centrifugation at 1,000 rpm for 10 min at 25 °C, erythrocytes were lysed by hypotonic solution and the cell pellets were washed twice with DMEM medium. The cells were resuspended in complete DMEM medium and the cell number was adjusted to 10^6 cell/mL. The viability of splenocytes was determined by the MTT dye technique. The rest of the procedure was same as mentioned above in the L929 cell line test.

The relative cell proliferation was measured by the following formula:

$$Rp = A_{test}/A_{control} \quad (2)$$

where, Rp—relative cell proliferation, A_{test}—absorbance of the test sample and $A_{control}$—absorbance of the control.

Results and discussion

Contact angle measurement

The contact angle of the goat blood-in-air over the ester films was found to be 57.8 ± 1.78° with the readings varying from 56 to 60°. This indicates the hydrophilic nature of the ester film. The hydrophilic nature of the esterified product may be accounted for to the presence of free amino groups. The lower the contact angle the higher is the hydrophilicity. Hydrophilicity has been suggested to be a key determinant of cell attachment [22]. Hydrophilicity is known to influence the adsorption of blood proteins and through these proteins to regulate a variety of cell behaviors such as cellular attachment. Although no relationship seems to exist between the wettability of the surface and the thromboresistance, contact angles have been used to characterize nonthrombogenic surfaces. Samples that produce small contact angles with blood are, in general, associated with good blood compatibility, whereas materials with large contact angles have poor compatibility [23]. From the above fact it can be assumed that the esterified product will be hemocompatible.

Hemocompatibility test

From the contact angle measurements it was found that the polymer developed was hydrophilic in nature indicating the polymer might be hemocompatible. To ascertain the same, hemocompatibility test was carried out. It was found that the % hemolysis due to the polymer was less than 5%, which suggests the sample as highly hemocompatible and the same could be tried as a moist wound dressing material (Table 1).

Water vapor transmission rate

In moist wound dressing WVTR plays an important role. Literatures suggest that materials for moist wound dressing should be moisture retentive i.e. WVTR of the dressing material should be <37 g/m^2/h. The WVTR through the membrane was found to be 0.074 g/m^2/h indicating that the membrane could be designated as moisture retentive and hence could be tried as a moist wound dressing material [24].

Measurement of diffusion coefficient

The desired hydrogels can be produced consistently with the technique outlined above. Typical variations of the concentration of SA in the two chambers during a single experiment are shown in Fig. 2. As can be expected, the concentration of the drug in donor decreased over time, while there was a corresponding increase in the concentration of drug in receptor. At any time, t, the concentration values in the two chambers can be used to calculate the diffusion coefficient, D, of the drug in the hydrogel from the following equation [25]:

$$D = 1/(\beta t) \times \ln[\{C_D(t) - C_R(t)\}/\{C_D(0) - C_R(0)\}] \quad (3)$$

with

$$\beta = (A_H/W_H)[(1/V_D) + (1/V_R)] \quad (4)$$

Table 1 Hemocompatibility test

	OD at 545 nm	% Hemolysis	Remarks
Positive	0.261	–	–
Negative	0.001	–	–
PVA-Gelatin hydrogel	0.0032	0.846	Highly hemocompatible

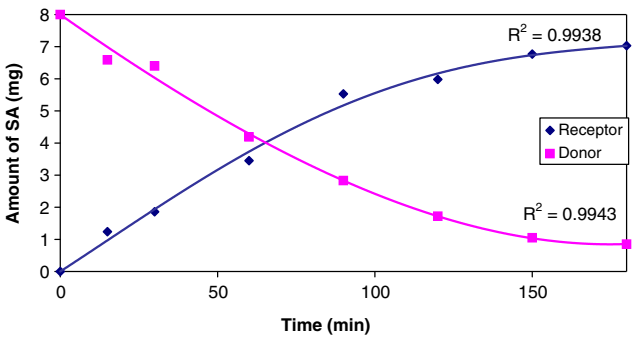


Fig. 2 Diffusion of SA through PVA–Gelatin Membrane

where: $C_D(0)$ = initial concentration of drug in donor; $C_R(0)$ = initial concentration of drug in receptor; $C_D(t)$ = concentration of drug in donor after time t; $C_R(t)$ = concentration of drug in receptor after time t; A_H = effective cross-sectional area of diffusion in the hydrogel sample; W_H = width of the hydrogel sample; V_D = Volume of donor sample; and V_R = Volume of receptor fluid.

A plot of $-\ln[\{C_D(t) - C_R(t)\}/\{C_D(0) - C_R(0)\}]$ (denoted by –lnX) with time yielded a straight line as shown in Fig. 3. The slope of this line can be used to calculate the diffusion coefficient, D as indicated in Eq. 3.

The diffusion coefficient of SA through the membrane was found to be 1.49×10^{-5} cm^2/s.

Typical variations of the concentration of gatifloxacin in the two chambers during another experimentation are shown in Fig. 4.

A plot of $-\ln[\{C_D(t) - C_R(t)\}/\{C_D(0) - C_R(0)\}]$ (denoted by –lnX) with time yielded a straight line as shown in Fig. 5. The slope of this line can be used to calculate the diffusion coefficient, D as indicated in Eq. 3.

The diffusion coefficient of gatifloxacin through the membrane was found to be 3.97×10^{-6} cm^2/s.

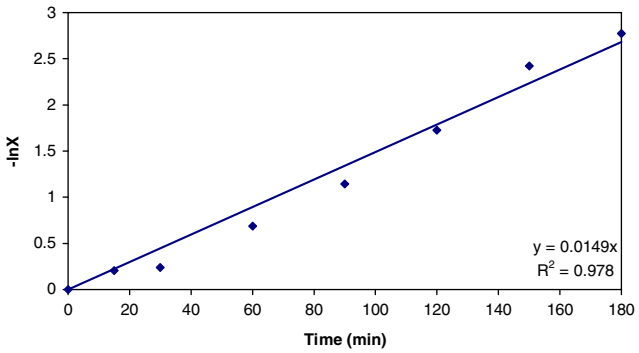


Fig. 3 –lnX Vs Time plot, whose slope was used for determining Diffusion coefficient (D) of SA through the hydrogel membrane

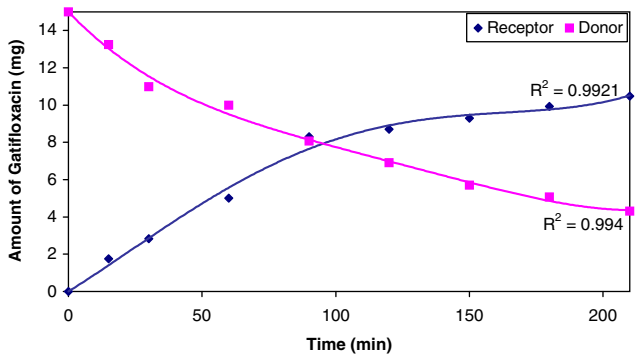


Fig. 4 Diffusion of gatifloxacin through PVA–Gelatin Membrane

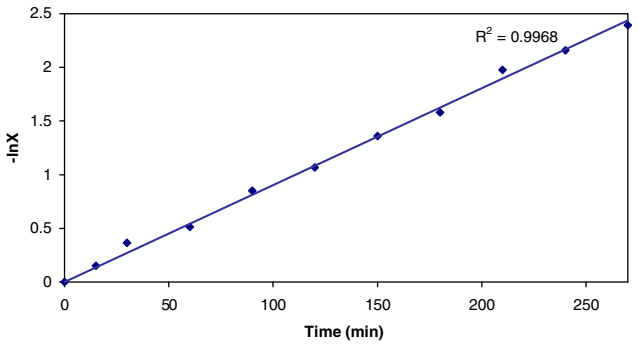
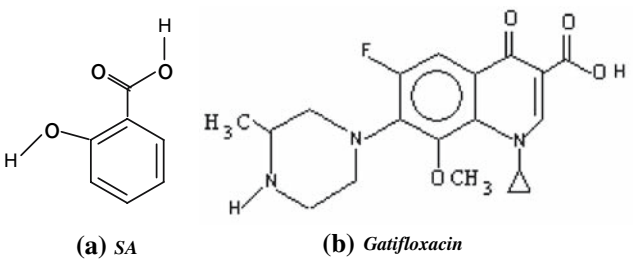


Fig. 5 –lnX Vs Time plot, whose slope was used for determining Diffusion coefficient (D) of gatifloxacin thriugh the hydrogel membrane

SA is an anionic drug while gatifloxacin is cationic in nature. Both the drugs are water-soluble. The molecular weight of SA and gatifloxacin are 138.1 and 384.4, respectively (Fig. 6). The results suggest that as the molecular weight of the drug increases, its diffusion coefficient through the hydrogel membrane decreases. In other words, it can be quoted that as the molecular size increases the diffusion coefficient of the drug decreases. Thus the membrane is permeable to both anionic and cationic drugs.

MTT Assay

MTT assay was performed in triplicate to measure changes in the viability of L929 cells and mice splenocytes after incubation with the membrane. The MTT assay is a quick effective method for testing mitochondrial impairment and correlates quite well with cell proliferation. In recent years it has been frequently used as a preliminary screen for the evaluation of in vitro cytotoxicity of polymeric components. The relative cell proliferations of the membrane were found to be 2.37 and 2.38 for L929 fibroblast cell line and mice splenocytes respectively. These results indicate that the membrane is biocompatible. From this it can be assumed that the composite membranes will show wound-healing activity because in wound healing fibroblast proliferation plays an important role. Since the membrane is supporting splenocyte proliferation, it can also be used as a



(a) *SA* **(b)** *Gatifloxacin*

Fig. 6 Structure of the drugs used for determining the diffusion coefficient through the hydrogel membrane

Table 2 Relative proliferations of L929 fibroblast and mice splenocyte cells over the ester membrane

Cells Used	Samples	Average (OD)	±SD	Rp
L929	Control	0.338	0.019	–
	Sample	0.801	0.01	2.37
Splenocytes	Control	0.042	0.004	–
	Sample	0.1	0.005	2.38

support structure for spleen after spleen surgery and/or trauma (Table 2).

Conclusion

PVA–gelatin membrane prepared by esterification reaction of hydroxyl group of PVA with carboxyl group of gelatin presents adequate characteristics to be used as a matrix for drug delivery and as a wound dressing material. The hydrogel was found to be hemocompatible and moisture retentive. The diffusion studies of SA and gatifloxacin through the membrane indicated the permeability of the hydrogel towards anionic and cationic drugs. However, the permeability of low molecular weight drug appears to be better. The membrane developed was found to be compatible with the L929 fibroblast cell line and mice splenocytes and has a good potential to be used as wound dressings and support structure for spleen and liver after surgery or trauma. Since the hydrogel membrane allows solute diffusion it can be useful in delivering drug or nutrient or growth factors directly to the wound site by putting a swab over the hydrogel without removing the hydrogel from the wound site.

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