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Preparation and characterization of amidated pectin based hydrogels for drug delivery system

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Abstract In the current studies attempts were made to prepare hydrogels by chemical modification of pectin with ethanolamine (EA) in different proportions. Chemically modified pectin products were crosslinked with glutaraldehyde reagent for preparing hydrogels. The hydrogels were characterized by Fourier transform infrared spectroscopy (FTIR), organic elemental analysis, X-ray diffraction studies (XRD), swelling studies, biocompatibility and hemocompatibility studies. Mechanical properties of the prepared hydrogels were evaluated by tensile test. The hydrogels were loaded with salicylic acid (used as a model drug) and drug release studies were done in a modified Franz's diffusion cell. FTIR spectroscopy indicated the presence of primary and secondary amide absorption bands. XRD studies indicated increase in crystallinity in the hydrogels as compared to unmodified pectin. The degree of amidation (D_A) and molar and mass reaction yields (Y_M and Y_N) was calculated based on the results of organic elemental analysis. The hydrogels showed good water holding properties and were found to be compatible with B-16 melanoma cells & human blood.

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1 Introduction

Hydrogels are three-dimensional cross linked polymer chains, which has the ability to imbibe and hold water within the cross linked structures. They are used for various biomedical applications such as soft contact lenses, wound dressing, super-absorbents and drug delivery systems due to their hydrophilic nature [1, 2]. Pectin is one of the major constituents of citrus by products and has good gelling properties [3, 4]. Chemically, pectin is poly α 1–4-galacturonic acids, with varying degree of methylation of carboxylic acid residues [5]. Pectins with low degree of methylation forms gel in presence of multivalent ions whereas pectins with higher degree of methylation forms gel in acidic media with the addition of different sugars, e.g., sucrose or glucose [6]. Pectin has been used since long time by the researchers as a potential drug carrier for colon specific drug delivery [7–9]. Amidated pectins are low methoxyl pectins in which some of the carboxylic acid groups are amidated. The chemical modification of pectin (amidation, trans-eterification) is relatively easy as it does not require extreme conditions and the amide bond is sufficiently resistant to hydrolysis by acids or alkali. Also the yield of prepared *N*-alkyl amides of pectin is relatively high. In the present study attempts were made to chemically modify pectin with ethanolamine. The amidated products so produced were converted into hydrogels by crosslinking with glutaraldehyde (GA) in acidic medium. Attempts were also made to characterize the products.

2 Materials and methods

Pectin (MW ~ 30,000–100,000), methanol, glutaraldehyde (GA) 25% solution and salicylic acid (SA, a model

drug) were obtained from Loba-chemie indoaustranal Co. Mumbai, India. Ethanolamine was obtained from SRL research laboratories, Mumbai, India. Hydrochloric acid 35% pure was obtained from Merck Limited, Mumbai, India. Double distilled water was used throughout the study.

2.1 Synthesis of modified pectin

The reactions were carried out according to the method described earlier [10]. In short, accurately weighed pectin (0.2 moles) was dispersed in 50 mL methanol and was transferred to a 250 mL three-necked flask. Ethanolamine (0.08 moles) was dissolved in a methanol (50 mL) and the resulting solution was gradually added to the pectin dispersion with continuous stirring. The temperature of the reaction mixture was maintained at 25 °C for 96 h. After 96 h the dispersion was filtered to obtain the product. The product so obtained was washed with 0.1 M HCl in ethanol–water mixture (1:1 ratio, v/v) to convert free carboxylic group into protonated form. Finally, the product was washed with 40% (v/v) ethanol until it showed a negative reaction to chloride, followed by drying at 60 °C. The product so obtained was named as EPH-1. Similarly, EPH-2, EPH-3, EPH-4 were synthesized having ethanolamine in concentrations of 0.16, 0.24 and 0.32 moles (Table 1). The EPH so obtained was used for the preparation of hydrogel membranes using glutaraldehyde reagent (1 mL + 0.2 mL Hcl) as crosslinking agent (Fig. 1).

2.2 Preparation of EPH hydrogels

10% w/v solutions of EPHs in water were prepared by dissolving 2 g of EPHs in 20 mL of distilled water. About 1 mL of glutaraldehyde reagent (1 mL GA + 0.2 mL HCl) was added to each of the EPHs solutions, were kept at stirring at room temperature. The homogeneous dispersions so obtained were converted into membranes by conventional solution casting method. The membranes were washed thoroughly with double distilled water. The membranes so obtained were named as EPH-1, EPH-2, EPH-3 and EPH-4, respectively.

Table 1 Compositions of pectin and ethanolamine (EA) used for the preparation of EPH membranes

Sample identity	Pectin (moles)	Ethanolamine (moles)	D_A (%)
EPH-1	0.02	0.08	22.46
EPH-2	0.02	0.16	33.0
EPH-3	0.02	0.24	59.61
EPH-4	0.02	0.32	68.83

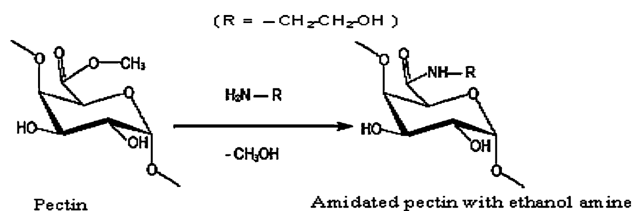

Fig. 1 Reaction mechanism of amidation of pectin with ethanolamine [10]

2.3 Characterization of EPH hydrogels

2.3.1 FTIR spectral analysis

The FTIR spectra of pectin and EPH were taken in the frequency range of 4,000–400 cm^{-1} as attenuated total reflectance (ATR) technique with the help of FTIR spectrophotometer (NEXUS-870, Thermo Nicolet Corporation).

2.3.2 Organic elemental analysis

The estimation of only three elements, i.e., carbon, hydrogen and nitrogen was done in 2400 series II CHN analyzer (Perkin–Elmer).

2.3.3 X-ray diffraction studies

X-ray diffraction studies of the raw materials and the products were done using Cu Kα radiation generated at 40 kV and 40 mA (XRD-PW 1700, Philips, USA). The samples were scanned in the 10°–70° 2θ range.

2.3.4 Tensile studies

The tensile strength of the EPH membranes were done using Hounsfield H10KS tensile testing machine:

(a) Cross-Head Speed: 12.5 mm/min
(b) Temperature: 18 °C and
(c) Relative Humidity: 60% RH.

2.4 Drug release profile of SA from EPH hydrogel

Cross-linked EPH were used for the drug release study. Salicylic acid (SA) was used as model drug and was incorporated in the cross linked hydrogel by diffusion method. EPH membranes were immersed for 5 h in SA solution in ethanol. The drug loaded EPH were washed

with distilled water to remove the drug, which adhered to the surface of hydrogel. The release study was carried out at pH 7.4 (Temperature = 37 ± 1 °C).

2.5 Cytotoxicity test

2.5.1 MTT assay

B16 melanoma cells were used to ensure the biocompatibility of EPH by MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5 diphenyltetrazolium bromide) assay [11]. Cells were seeded into a 96-well microplates at 3,000 cells per well. The medium was removed after 24 h of plating and fresh media containing different concentrations of polymers (1–100 μg/mL) were added. After incubation for 1 h the medium was discarded, the cells were washed twice with phosphate-buffered saline (PBS: 150 mM NaCl; 1.9 mM NaH2PO4; 8.1 mM Na2HPO4; pH 7.4) and 50 mL of 5 mg/mL MTT solution in PBS were added to each well. The plates were incubated 37 °C for 4 h and the formazan crystals were dissolved by adding 100 mL dimethyl sulfoxide (DMSO) to each well. The absorptions were measured in triplicate at 570 nm. Results were recorded as percentage absorbance relative to untreated control cells. The cytotoxicity assay results were used to calculate cell viability after incubation with polymers as follows:

$$\%\,\text{Cell Viability} = \frac{X}{X_c} \times 100 \tag{1}$$

where X is the absorbance in a well containing a particular polymer concentration and X_c is the absorbance for untreated control cells.

2.6 Haemocompatibility test

The hemolysis tests were carried out broadly on the basis of ASTM standard [12]. The hemolysis percentage is defined as:

$$\%\,\text{Hemolysis} = \frac{OD_{\text{test}} - OD_{\text{negetive}}}{OD_{\text{positive}} - OD_{\text{negetive}}} \times 100 \tag{2}$$

3 Results and discussion

3.1 Elemental analysis

The results of elemental analysis (Table 2) indicated absence of nitrogen in pectin but the nitrogen content increased from EPH-1 to EPH-4. The degree of amidation (D_A) also increased from EPH-1 to EPH-4. This could be accounted to incorporation of amide group in the pectin structure. The degree of amidation (D_A), the mass and molar yield of the reaction was calculated based on elemental analysis results, according to the following equations [15].

$$\begin{aligned} D_A &= \frac{M_N}{M_C}\left[6 + \left(\frac{73}{100}\right) + K - 1\frac{M_N}{14}\right] \times 100 \\ Y_M &= \frac{M_N \times M_A}{14} \\ Y_N &= \frac{D_A}{73} \times 100 \end{aligned} \tag{3}$$

where D_A is the degree of amidation, Y_M the mass yield of the reaction, i.e., the relative mass of bonded amine in reaction product, the molar yield of the reaction, i.e., the relative content of ester groups substituted by amine (%), M_N the nitrogen content (%) and M_C the carbon content, M_A the molar mass of amine(g mol^{-1}), 12 the carbon atomic mass (g mol^{-1}), 14 the nitrogen atomic mass (g mol^{-1}), 6 the sum of carbon in galacturonic unit, K the number of carbons in amine molecule and 73 is the degree of methylation (D_M) of original pectin.

3.2 FTIR characterization

The spectrum of pectin (Fig. 2a) indicated peak at 3402 cm^{-1} due to the stretching of –OH groups. The peak at 2932 cm^{-1} indicated C–H stretching vibration. The peaks at 1,460 and 1,377 cm^{-1} could be assigned to –CH_2 scissoring and –OH bending vibration peak respectively. The peak at 1,017 cm^{-1} suggested –CH–O–CH– stretching. The peak at 1,165 cm^{-1} suggested the presence of –CH–OH in aliphatic cyclic secondary alcohol. FTIR spectrum of EPH-1 (Fig. 2b) indicated peaks at 1,666 cm^{-1} (amide I) and 1,595 cm^{-1} (amide II) and absence of carboxylate stretching. The peak at 2,926 cm^{-1} indicated the presence of symmetric C–H stretching vibrations of methylene groups. This can be accounted to the increase of C–H bond content after amidation reaction. The peak at 1409 cm^{-1} suggested –CH bending of –CH_2 group. The peak at 1085 cm^{-1} suggested –OH bending, 1041 cm^{-1} indicated the presence of secondary alcohol (characteristic peak of –CH–OH in cyclic alcohol C–O stretch).

3.3 X-ray diffraction study

The Fig. 3a, b shows XRD pattern of the pectin and EPH-1 hydrogel respectively. X-ray diffractogram of the EPH-1 hydrogel showed three intense peaks at 46.13°, 39.89°, 22.06° 2θ, where as the diffractogram of pectin peak indicated peaks at ~13.34°, 5.03°, 2θ. The diffractogram of the pectin & EPH-1 hydrogel indicated that there is an increased in crystallinity of pectin after modification.

Table 2 Results of elemental analysis and degree of amidation (D_A), mass yield of the reaction (Y_M) and molar yield of the reaction (Y_N) of EAMP samples

Sample Identity	M_C (%)	M_H (%)	M_N (%)	D_A (%)	D_M (%)	Y_M (%)	Y_N (%)
Pectin	36.27	5.85	0.39	–	73	–	–
EPH-1	33.08	6.21	1.11	22.46	31	0.39	6.84
EPH-2	38.31	5.56	2.17	33.0	33	1.55	13.69
EPH-3	32.56	7.25	2.83	59.61	34	3.03	20.54
EPH-4	31.55	7.32	3.13	68.83	32	4.47	27.39

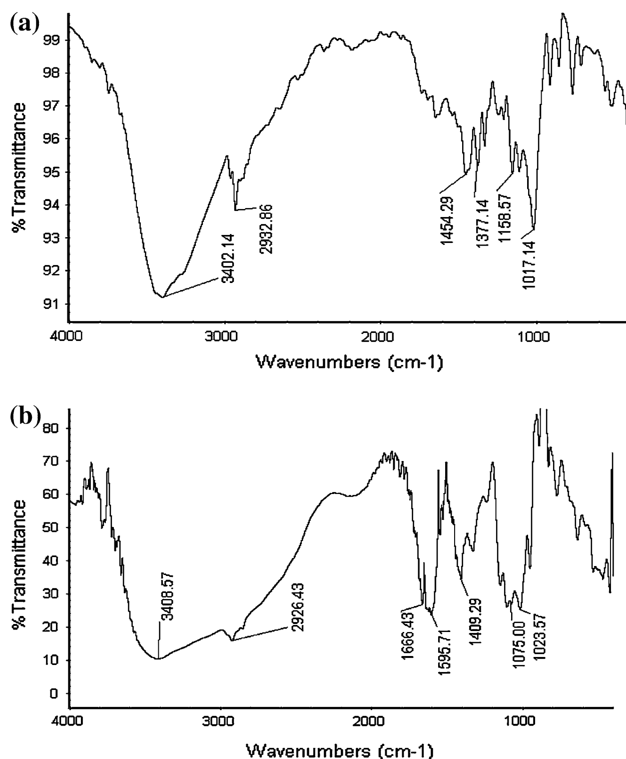

Fig. 2 FTIR spectrum of (**a**) Pectin; (**b**) EPH-1

3.4 Tensile strength of the EPH hydrogel membranes

Figure 4 shows the tensile strength of prepared EPH membranes. EPH-1, EPH-2 EPH-3, and EPH-4 had almost comparable tensile strengths. The intra-molecular hydrogen bonding in EPH-1, EPH-2, EPH-3 and EPH-4 were minimal which resulted in lower tensile strengths.

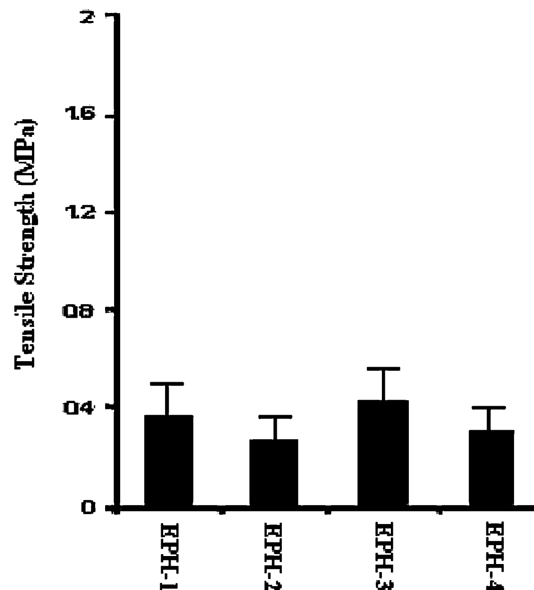

Fig. 4 Tensile strength of EPH membranes

3.5 Drug release studies

Figure 5 shows the SA release profile from the EPH membranes at pH 7.4. It could be observed that the SA release from the hydrogel membranes was maximum in EPH-4 followed by EPH-3, EPH-2 and EPH-1. This could

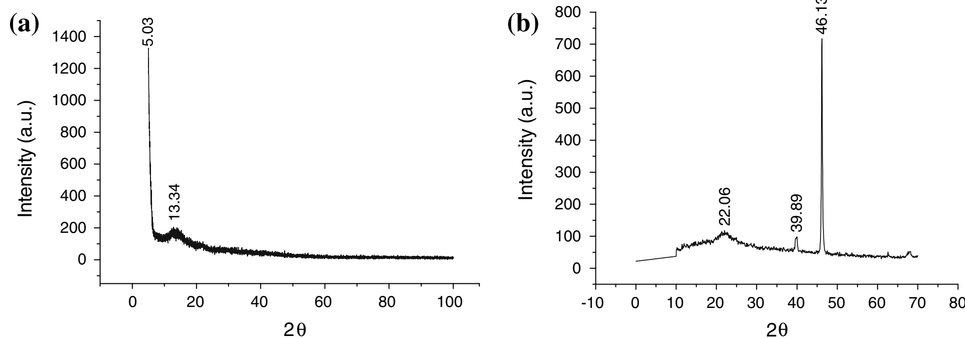

Fig. 3 XRD patterns of (**a**) Pectin; (**b**) EPH-1

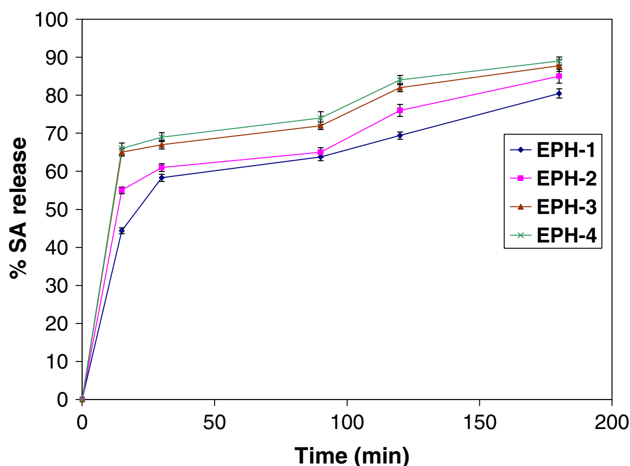

Fig. 5 SA release pattern from the EPH hydrogel membranes at pH7.4

be accounted to the fact that the gel structure swells more with the increase in the amide group in the hydrogel membrane. As the % of amide group is higher in the EPH-4 membrane it swells more readily allowing rapid diffusion of the buffer into the gel structure and hence rapid release of the drug.

3.6 Drug release kinetics from the hydrogel

The release kinetics of the drug from hydrogels Higuchian kinetics, thus the drug release from the hydrogels are diffusion-controlled (Fig 6). The rate of release of a drug dispersed as a solid in an inert matrix has been described by Higuchi [13–15]. For the purposes of data treatment the above model is depicted by the following equation;

$$M = kt^{1/2}$$

where M is the mass of the drug released per unit area, k is a constant, so that a plot of amount of drug released versus the square root of time, t, should be linear if the release of the drug from the matrix is diffusion controlled.

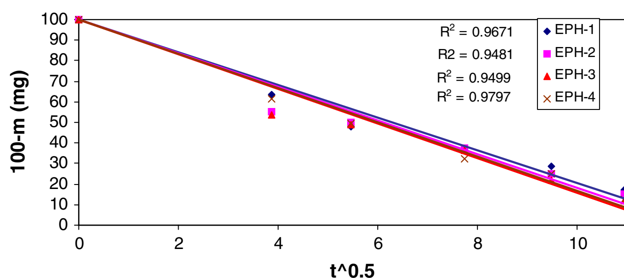

Fig. 6 Drug release kinetics from the hydrogels

Table 3 Hemocompatibility study of EPH membranes

S.No	Hydrogel compositions	Hemolysis (%)	Remarks
1	1:1	3.39	Highly hemocompatible
2	1:2	3.57	
3	1:3	3.66	
4	1:4	3.96	

3.7 Haemocompatibility study

Haemocompatibility results of the EPH membranes revealed highly haemocompatible nature of the membranes (Table 3).

4 Cytotoxicity study of EPH membranes

The MTT assay was used to investigate changes in cell viability in the presence of the polymers. Reduction of MTT by cells indicates mitochondrial activity of live cells and is often related to the cell viability. The EPH hydrogel didn't induced significant cytotoxic effects even at the higher concentrations used (Fig. 7), EPH is a biocompatible hydrogel.

5 Conclusions

In the current work EPH has been prepared by the amino de- alkoxylation (aminolysis) of pectin by reacting with ethanolamine. The prepared EPH was cross-linked with GA. The structural changes in the modified pectin were investigated by FTIR spectroscopy and organic elemental analysis. FTIR spectra of EPH indicated amide I and amide II vibration peaks along with the N-alkyl peak, which was supported by organic elemental analysis. Many bioactive molecules viz protein, peptides, enzymes or drugs could be

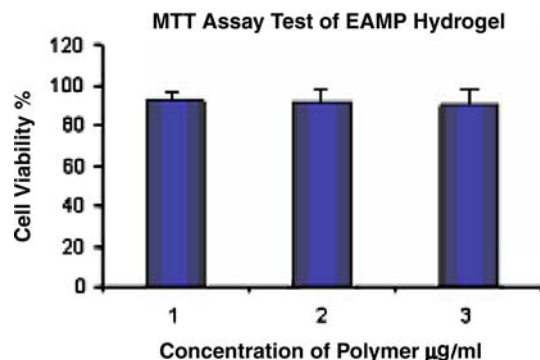

Fig. 7 Cytotoxicity of EPH hydrogel (1 = 1 µg/L, 2 = 10 µg/L, 3 = 100 µg/L)

immobilized on the surface of pectin surface via amidation. The drug release from the hydrogel matrix followed Higuchian's kinetics. From the in vitro studies it is evident that EPH membranes could be tried as a matrix for trans-dermal drug delivery systems and as a wound dressing material.

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