Preparation of Genipin-Fixed Agarose Hydrogel

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ABSTRACT: Genipin, a naturally occurring crosslinker, was employed to modify agarose in aqueous medium at pH ~ 7 at ambient conditions. The physical and rheological properties were studied. The genipin-fixed agarose hydrogel (0.8 wt % genipin with respect to the polysaccharide) having the maximum swelling capacity was obtained after 85-h crosslinking reaction. The reaction mixture developed a dark blue color with the passage of time, indicating thereby the progress of the crosslinking reaction in presence of genipin. The maximum swelling of the genipin-fixed agarose hydrogel in acidic medium at pH 1.2 was ~ 48 g/g, whereas the parent polysaccharide agarose achieved equilibrium swelling state at 6 g/g. The genipin-fixed agarose showed 30% weight loss in Ringer's solution, while the agarose polysaccharide exhibited ~ 50% weight loss in the same medium, both after 60 days. The thermogravimetric analysis studies revealed enhanced thermal stability of the genipin-fixed agarose hydrogel. In view of the enhanced stability and swelling capacity of the genipin-fixed agarose compared to agarose, the value added polysaccharide may be useful in new applications as super absorbents and in biomedical applications. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 290–296, 2007

Key words: agarose; genipin; crosslinked hydrogel polymer; absorbent

INTRODUCTION

Hydrophilic gels are in increasing demand in the biomedical and pharmaceutical applications because of their biocompatibility.[1–3] Most of these applications in the past have made use of synthetic water soluble polymers (WSP) such as polyacrylic acids, polyacrylamides, polyethelene oxide, polyvinyl alcohols, and polyvinyl pyrrolidones. Subsequently, these hydrogels were modified by blending some natural polymers with WSP.[4] The resultant hydrogels exhibit different properties than those of the original polymers.[5,6] The improved properties of the hydrogels were harnessed either as super absorbents or as controlled delivery systems. The natural polymers that have been widely used for this purpose are cellulose, starch, chitin, carrageenan, agar, and alginates.[7] Genipin is a naturally occurring crosslinking agent having much less toxicity and widely used in herbal medicine,[8] and the dark blue pigments obtained by its spontaneous reaction with amino acids or proteins have been used in the fabrication of food dyes.[9] It was reported that porcine pericardia crosslinked with genipin led to the formation of stable crosslinked products.[10] It has also been reported that the gelatin-derived bioadhesives display higher biocompatibility and less cytotoxicity when crosslinked with genipin than with other agents, such as formaldehyde, glutaraldehyde, and epoxy compounds.[11,12] Gerard et al.[13] has described estimation of amino acid concentrations in various polymeric matrices including agar, agarose, and carrageenan. Protein levels in bacteriological agar were estimated on the basis of nitrogen content of the polysaccharide.[14] Agarose is a hydrophilic polymer and widely used in biomedicinal applications and bioengineering. The chemical structure of genipin [Fig. 1(a)] and the basic disaccharide repeating units of agarose, (1 → 3) linked β-D-galactose and (1 → 4) linked α-L-3,6-anhydrogalactose [Fig. 1(b)], are reported in the literatures.[15,16]

In this article we report the effect of genipin, a naturally occurring crosslinking agent, on agarose and preparation of absorbent genipin-fixed agarose hydrogel, which is thermally more stable and degrades slowly in Ringer's solution compared to agarose. The product was characterized by thermogravimetric analysis (TGA), gelation degree, swelling ability, and rate of degradation studies. The reaction mechanism of genipin with amino groups and schematic illustration (Fig. 2) of the formation of genipin-fixed agarose network has been proposed.[15]

EXPERIMENTAL

Materials and methods

The phycocolloid, agarose, was extracted from the red seaweed *Gracilaria dura* occurring in Indian waters. Genipin was purchased from Challenge Bioproducts Co., Taiwan. Iso-propanol (Laboratory Reagent grade)

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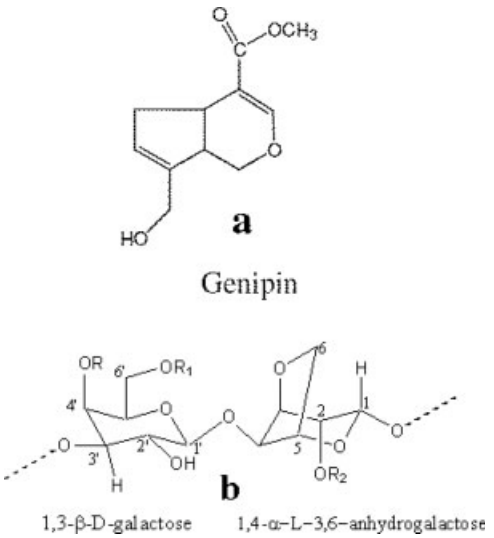


Figure 1 Structure of (a) genipin and (b) agarose (R = H or Me, R_1 = H or Me, R_2 = H or Me).

was procured from Ranbaxy Chemicals, Mohali (Punjab), India.

Preparation of agarose

Agarose was prepared from the red seaweed *Gracilaria dura* using an improved procedure described by Siddhanta et al., 2004.[17–19]

Preparation of genipin-fixed agarose hydrogels

In a typical batch, the agarose sol was prepared by dissolving 2 g of agarose in 50 mL of distilled water at 120°C for 20 min in an autoclave. A stock solution (10%) of genipin was prepared in 10 mL of 60% aqueous ethanol. The aqueous solution of the agarose was then mixed with different volumes of the genipin stock solution at 40°C to obtain the final polymer agarose–genipin mixture with weight percentages of genipin lying in the range 0.05–1.5 wt %. The homogeneous viscous solutions were kept at room temperature (30°C) and allowed to react for different durations e.g., 10, 20, 30, 40, 50, 60, 65, 70, 75, 80, 85, 90, 95, and 100 h, in 14 different experiments. The reaction mixture started assuming light blue color after 120 min and the color intensified with the passage of time becoming deep blue in color after 60 h. The gelled reaction mixture in each experiment was worked up to obtain the genipin-fixed agarose hydrogel by dehydrating the gel with isopropanol (1:2.5 w/w) for 24 h. After dehydration, the gel sample was air-dried followed by drying at 50°C for 2 h.

Gelation degree

The gelation degree G was calculated adopting the method reported by Lendlein et al.,[20] using eq. (1).

$$G = m_d/m_{\text{iso}} \tag{1}$$

where, m_d is the dry weight of the gel and m_{iso} is the weight of isolated gel.

A known weight of the dry genipin-fixed agarose hydrogel was taken, which was put into the different pH media (e.g., pH 1.2, 7.0, and 12.5) for equal time duration of 10 h, in separate experiments. The weight of the isolated gel samples were determined (m_{iso}). The isolated gel samples were dehydrated with a 100-fold excess of isopropanol overnight, washed with isopropanol carefully, and dried at room temperature under reduced pressure, and the sample was weighed again (m_d).

Swelling ratio measurements

The swelling ratio of agarose and genipin-fixed agarose with different weight percentage 0.5, 0.8, and 1.0% of genipin (with respect to the parent polysac-

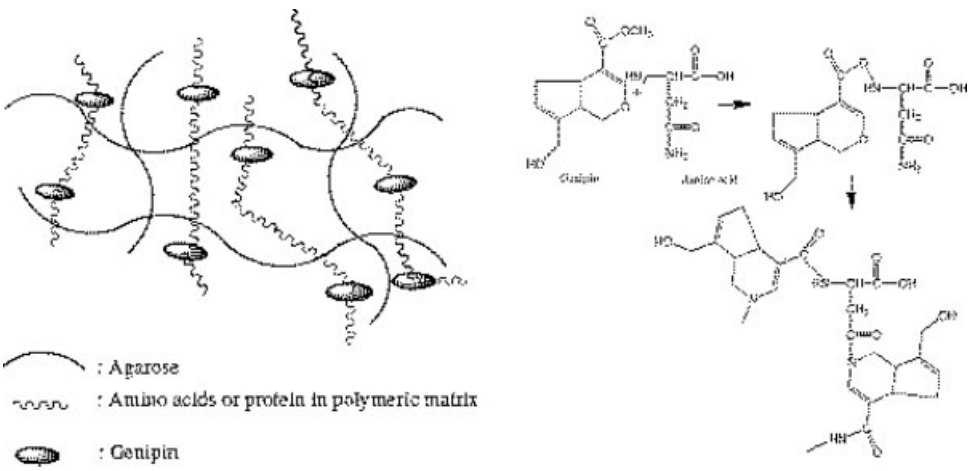


Figure 2 Schematic illustration of the formation of genipin-fixed agarose.

charide) were measured in this study. In the swelling measurements, the dry hydrogel was weighed (W_0) and immersed in aqueous media, having different pHs e.g., 1.2, 7.0, and 12.5, separately. After the designated soaking time had elapsed, the wet samples were wiped dry with filter paper to remove excess liquid, and weighed (W_t). The swelling ratio ΔW (%) was calculated using eq. (2).

$$\Delta W(\%) = \frac{W_t - W_0 \times 100}{W_0} \quad (2)$$

Degradation rate measurement

The degradation rate patterns of genipin-fixed agarose and agarose were measured in Ringer's solution (prepared in our lab: sodium chloride, 8.6 mg ml^{-1}; potassium chloride, 0.3 mg ml^{-1}, and calcium chloride dehydrate, 0.33 mg ml^{-1} under aseptic conditions), a reconstituted physiological medium.[21] The agarose and genipin-fixed agarose hydrogels (having 0.5, 0.8, and 1.0 wt % of genipin) were weighed (W_0) and placed in a capped plastic aseptic tubes with 25 mL Ringer's solution. The ion concentrations are similar to those in human physiological environments. The test samples were immersed in the Ringer's solution, as simulated body fluid, to measure the rate of degradation *in vitro*. Samples were incubated at 37°C in an incubator, after soaking for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 days, and all the mixtures were drawn from the Ringer's solution, dried to remove water, and weighed (W_t). The weight loss ratio ΔW (%) was then calculated using eq. (3).[21]

$$\Delta W(\%) = \frac{(W_0 - W_t) \times 100}{W_0} \quad (3)$$

Optimization of the crosslinking reaction time

Optimization of the reaction time was done on the basis of swelling capacity of the genipin-fixed agarose samples in the different pHs solutions.

Optimization of genipin quantity

Optimization of the genipin concentration was done on the basis of swelling capacity of the genipin-fixed agarose samples in the different pH solutions.

Characteristics of genipin-fixed agarose hydrogel

The agarose and genipin-fixed agarose hydrogel were characterized using different techniques. Optical microscopy was recorded on an Olympus model SZH 10, Japan, with 70× magnification and thermal analysis (TGA were done on a TGA Toledo Mettler TGA System, Switzerland). Apparent viscosity was measured on a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02,072) using Spindle No.1 at rpm 60. UV–vis spectra were recorded on a Varian CARY 500 Scan UV-Vis-NIR spectrophotometer. Optical rotation was measured on a Rudolph Digi pol – 781 Polarimeter (Rudolph Instruments, NJ) in 0.025% aqueous solution at 45°C.

Elemental analysis and protein estimation

The elemental analyses were done on a PerkinElmer-2400, CHNS/O analyzer and total nitrogen was estimated by Kjeldahl method[22] on a KEL PLUS-KES 20L Digestion unit attached to a KEL PLUS-CLASSIC DX Distillation unit (M/s PELICAN Equipments, Chennai, India). Crude protein content was calculated multiplying the nitrogen content by the approximate factor 6.25.[22]

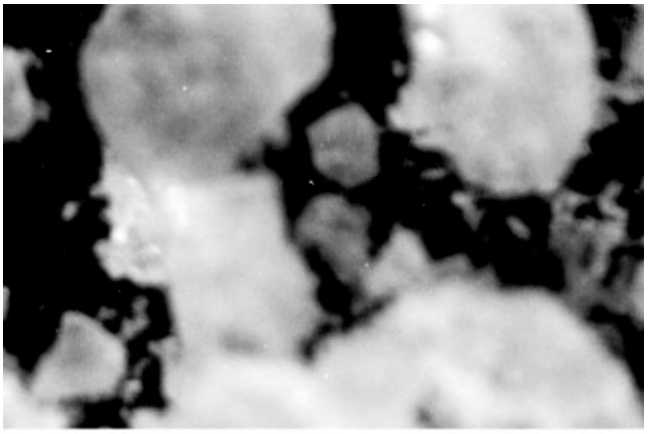
Rheological measurements

Dynamic rheological measurements were done on a rheometer (RS1, HAAKE Instruments, Karlsruhe, Germany). The measuring geometries selected were a cone/plate (60 mm diameter, 1° rad angle) for measurements in 1% sols taking 1 mL sol on to the plate of the rheometer, for measurements in the sol, prepared from genipin-crosslinked agarose hydrogel with 0.8 wt % genipin and compared with agarose. Viscosities at varying shear rate were studied at 45°C. Oscillation measurements were carried out in controlled deformation mode with 0.05% strain and plate/plate geometry (35 mm dia) was used. The temperature was maintained using the DC50 water circulator. Rheological data presented are means of three replicate measurements.

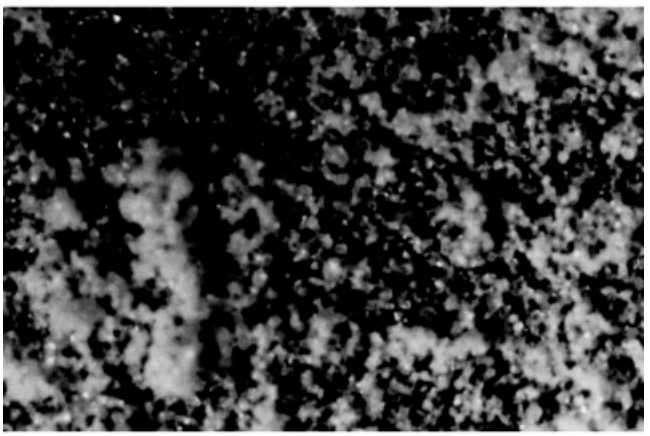
RESULTS AND DISCUSSION

Optical microscopy

The genipin-crosslinked agarose hydrogels were blue in appearance caused by the crosslinking reaction between genipin and agarose. The agarose and genipin were colorless before mixing together, but light blue color appeared after 120 min of mixing and the mixture became dark blue after 60 h. Optical micrograph of the powdered product, blue in color, is given in Figure 3.[23] The protein content in parent agarose was estimated to be 0.25%. Gerard et al.[13] estimated the amino acid concentration of glycine and amino acids (excluding glycine) to be in the range of 290 ng/mg (0.029%) and 376 ng/mg (0.037%), respectively. It is, therefore, apparent that genipin reacted with the amino acids present in the polysaccharide affording the crosslinked product. It was further observed that genipin reacted with gelatin producing dark blue color within minutes of mixing, whereas in the present investigation it took about 60 h to pro-



(a)



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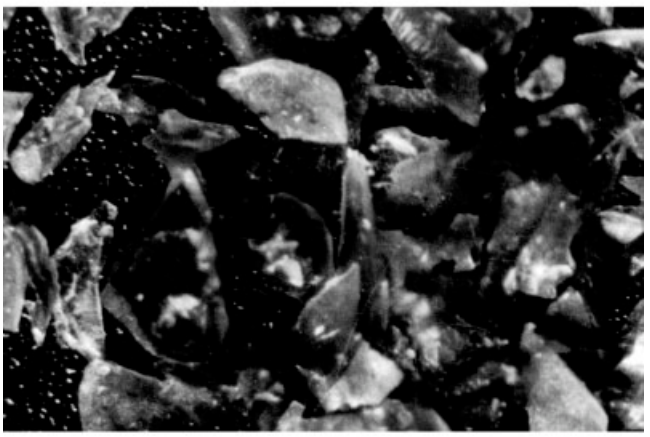

(c)

Figure 3 Optical micrographs of (a) genipin, (b) agarose, and (c) genipin-fixed agarose (with 0.8 wt % genipin).

duce dark blue color indicating that the amino acids present in agarose in trace amounts.

Gelation degree

The gelation degree (G) of the nonmodified agarose decreased after crosslinking with genipin. The G values for agarose 0.l62, 0.169, and 0.2 were observed at 1.2, 7.0, and 12.5 pH media, respectively. The gelation degree of genipin-fixed agarose decreased significantly with optimum 0.8 wt % genipin to 0.023, 0.024, and 0.027 at 1.2, 7.0, and 12.5 pH media, respectively, (Fig. 4).

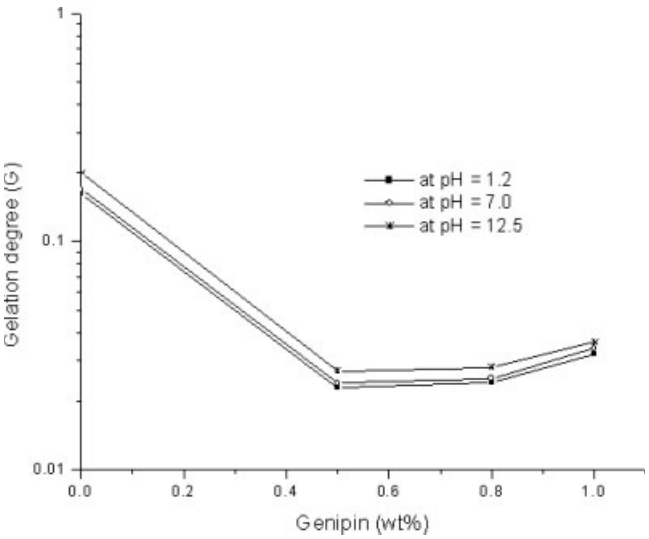

Figure 4 Dependence of degree of gelation degree on concentration of genipin in the different pH media.

Swelling behavior

The genipin-fixed agarose, obtained with 0.8 wt % genipin after 85 h crosslinking reaction time, showed the swelling ability in the following order in pH media 1.2 > 7.0 > 12.5, having maximum swelling ratios (%) 4800, 4300, and 3600 in the solutions of pHs 1.2, 7.0, and 12.5, respectively, [Table I and Fig. 5(a–c), 6 and 7]. On the other hand, the parent agarose exhibited swelling ratios (%) $\sim$ 600 in all the pH media, clearly indicating the improved network system in the genipin-fixed agarose hydrogel [Table I, Fig. 5(a–c)]. One important observation is the remarkable stability of the genipin-fixed agarose hydrogel in acidic pH, while in such acidity agarose gets readily depolymerized and dispersed [Fig. 5(a)]. Agarose swells up to $\sim$ 60 min and then achieves a steady value, whereas the product swells gradually

TABLE I
Equilibrium Swelling Ratios (%) of the Genipin-Fixed Agarose (with 0.0, 0.5, 0.8, and 1.0 wt % genipin) after $\sim$ 1100 min

Wt %	pH 1.2 [Fig. 5(a)]	pH 7.0 [Fig. 5(b)]	pH 12.5 [Fig. 5(c)]
0.0	600	600	500
0.5	3000	2900	2800
0.8	4300	4200	3700
1.0	4000	4000	3600

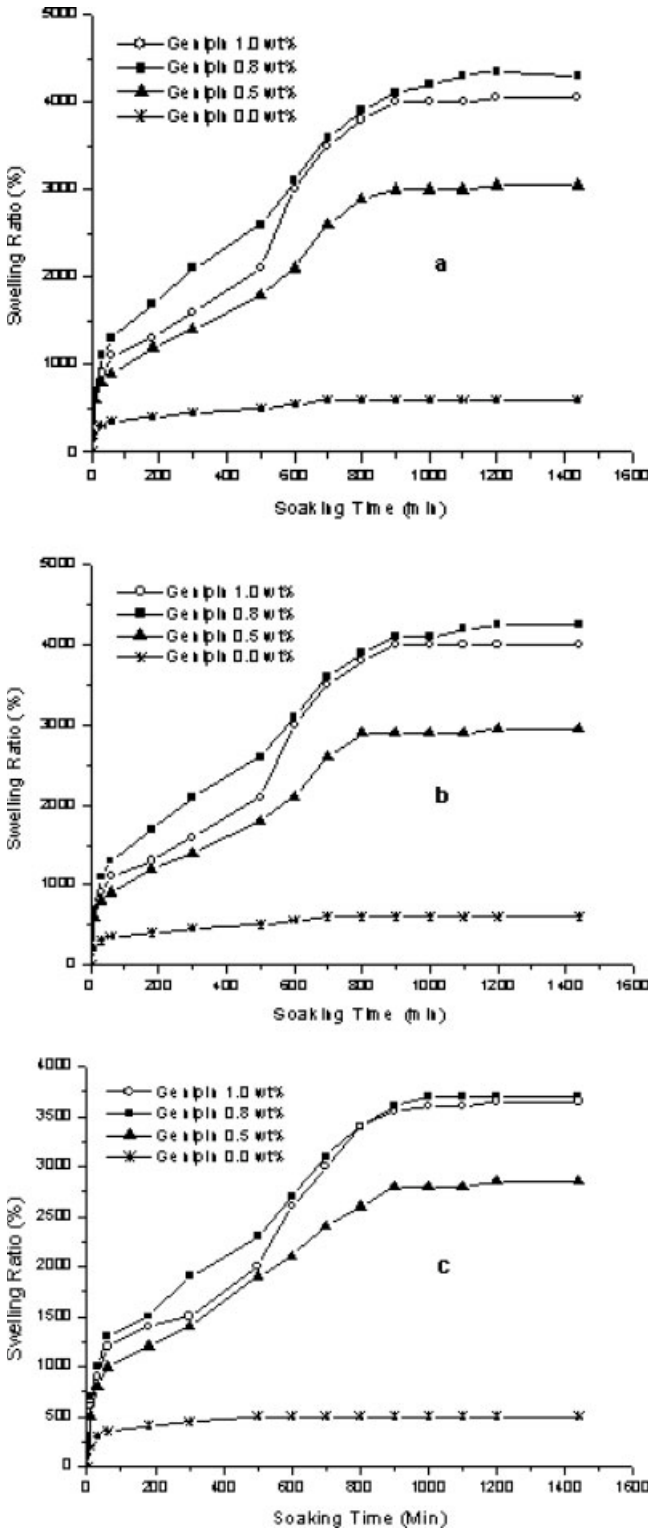

Figure 5 Swelling ratios of agarose and the different genipin-fixed agarose obtained after 85 h crosslinking reaction; soaking at (a) pH 1.2, (b) pH 7.0, and (c) pH 12.5.

up to ~ 1100 min and then achieves an equilibrium value, which was followed by very slow degradation only in pH 1.2 [Fig. 5(a)]. The swelling ratio of genipin-fixed agarose with crosslinking reaction time (Fig. 6)

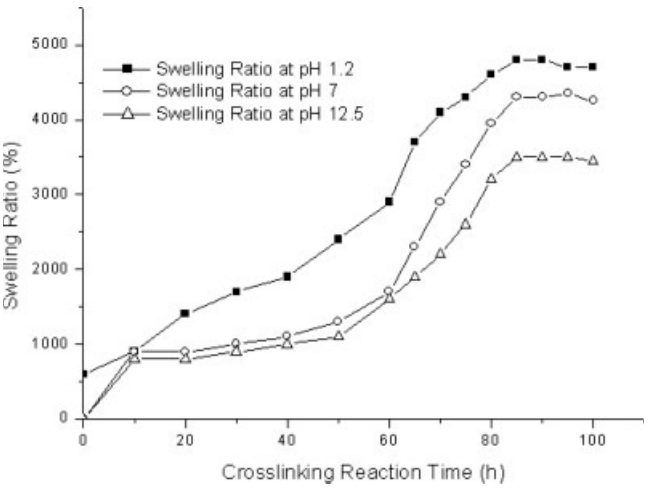

Figure 6 Effect of crosslinking reaction time on the swelling ratio of genipin-fixed agarose (with 0.8 wt % genipin).

and with concentration of genipin (Fig. 7), indicates that the minimum reaction time may be 85 h for agarose and genipin is 0.8 wt %. Thus, the swelling ratio of the crosslinked agarose had an inverse relationship with the gelation degree.

Degradation rate measurement

The mass loss ratio of nonmodified agarose and genipin-fixed agarose (with 1.0, 0.8, and 0.5 wt % genipin) was measured in Ringer's solution, wherein 50, 32, 33, and 38% mass loss was observed for agarose, genipin-fixed agarose (with 1.0 wt % genipin), genipin-fixed agarose (with 0.8 wt % genipin), and genipin-fixed agarose (with 0.5 wt % genipin), respectively, (Fig. 8). The mass loss ratio indicated that there are no significant variations in the mass loss between genipin-fixed agarose prepared with 1.0 and 0.8 wt % genipin.

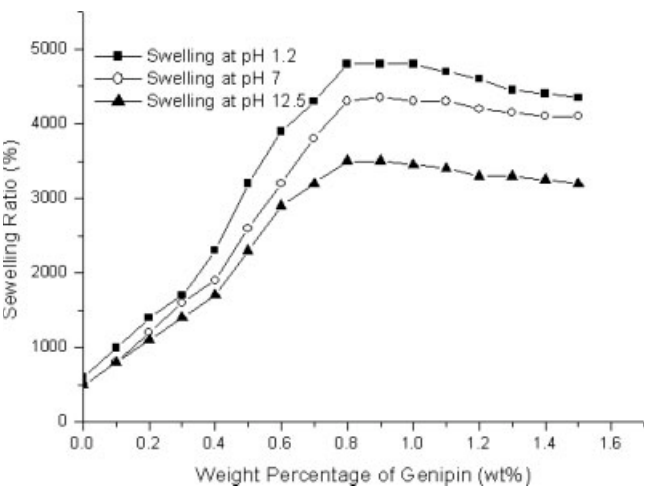

Figure 7 Effect of concentration of genipin on the swelling ratio of genipin-fixed agarose, after 85 h crosslinking reaction.

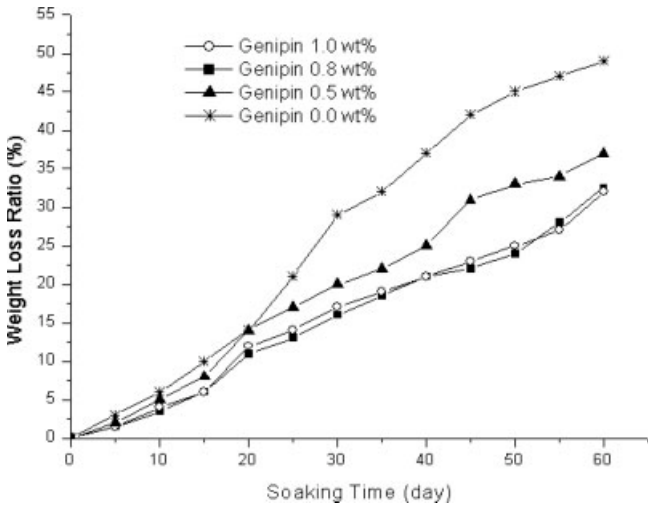

Figure 8 Effect of concentration of genipin on the weight loss ratio of agarose and genipin-fixed agarose in Ringer's solution, after 85 h crosslinking reaction.

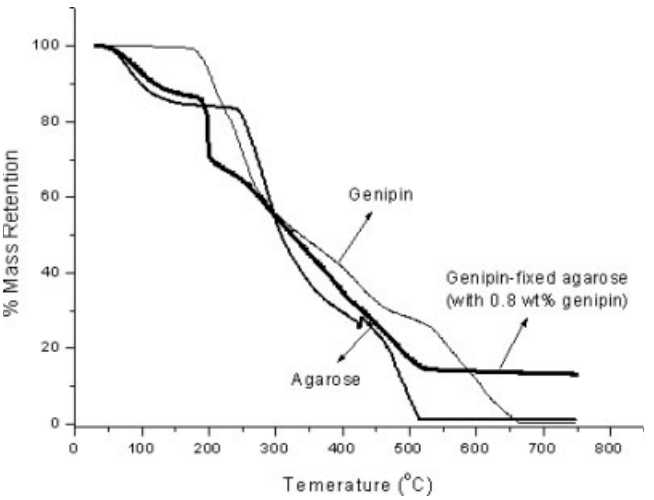

Figure 9 Thermogram (TGA) of agarose, genipin, and genipin-fixed agarose (with 0.8 wt % genipin).

TGA

The TGA curve for agarose, genipin, and genipin-fixed agarose polymer are shown in Figure 9. The TGA curve of agarose shows three stages of weight loss. The first weight loss (12%) stage between 30 and 110°C was due to the loss of water, the second weight loss (62%) stage between 240 and 350°C, and complete weight loss (100%) was observed between 350 and 520°C. The weight loss in genipin-fixed agarose polymer was also obtained in three stages. In the first stage weight loss was 10% between 30 and 121°C, in second stage weight loss was 30% between 180 and 250°C, and in third stage ~ 90% weight loss was observed up to 750°C. The latter shows enhance thermal stability of the product.

UV-spectroscopy and optical rotation

The specific rotation values of agarose and genipin were −21.6° and +111.1° respectively, while that of genipin-fixed agarose was −12.2°. This considerable change in the optical rotation also suggests substantial modification in the molecular geometry of the parent agarose that came about in the genipin-fixed polysaccharide. The UV spectra shows peak at 590 cm^{-1} due to reaction of genipin with the amino acid that is present in agarose giving rise to blue color.

Morphological analysis

The optical micrographs of genipin-fixed agarose was taken with 70× magnifications and compared with the parent agarose and genipin (Fig. 3). Optical micrograph of genipin-fixed agarose was dissimilar in morphology and in color with those of the parent polysaccharide. These suggest that the crosslinking reaction of genipin in agarose brought about transformation in the polysaccharide resulting in the changed morphology and color in the product.

Elemental analysis and protein estimation

The percentage of C, H, and N were 35.58%, 6.44%, and 0.0%. The CHN percentages remained unchanged in genipin-fixed agarose relative to unmodified agarose. The total nitrogen and protein values of agarose and genipin-fixed agarose were similar (0.07 and 0.4%, respectively) before crosslinking and after crosslinking with genipin, presumably because of the very low quantity of genipin that was involved in the crosslinking process with agarose.

Rheological measurement

Variations of dynamic viscosity with shear rate are summarized in Figure 10 with 1% sol of agarose and

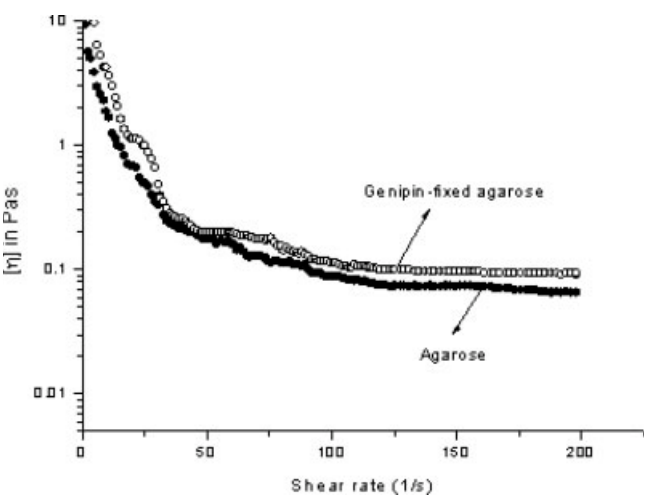

Figure 10 Variations in shear viscosities of agarose and genipin-fixed agarose hydrogel (with 0.8 wt % genipin).

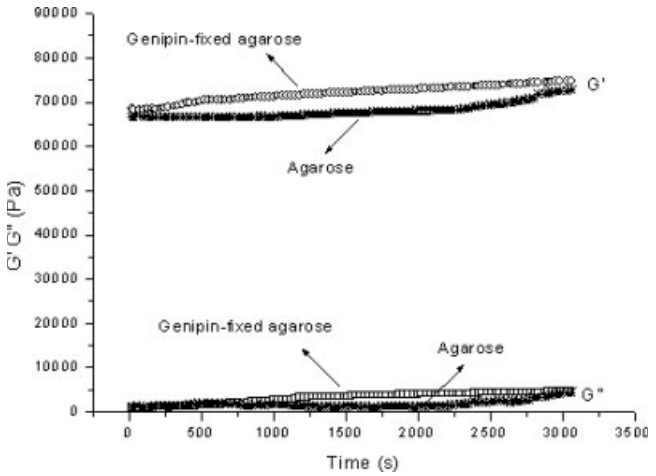

Figure 11 Time dependence of modulus ($G'G''$) of agarose and genipin-fixed agarose hydrogel (with 0.8 wt % genipin).

genipin-fixed agarose. It was observed that 1% sol of the genipin-fixed agarose hydrogel showed less gel-thinning behavior and high viscosity values than the sol of the parent agarose under applied shear rates. These values indicate enhanced network formation in the product facilitated by the crosslinker genipin. The stability of modulus during storage at 25°C was investigated and is depicted in Figure 11. The storage modulus values of the 1% gels of agarose and genipin-fixed agarose hydrogel. It was observed that the G' and G'' values for both samples slightly increased with increment of time. This observation shows the stability of the samples under stress for long time. It was also observed that the G' values for genipin-fixed agarose hydrogel was slightly higher than that of agarose.

CONCLUSIONS

In this study the effect of genipin, a naturally occurring crosslinker, on the properties of agarose has been demonstrated, and a value added product was prepared. Genipin imparted thermal stability and enhanced swelling ability, crystallinity, and lower gelation degree. The genipin-fixed agarose hydrogel exhibited superior absorbent property and stability in acidic solution at pH 1.2, lower degradation rate relative to the parent agarose. Thus, this naturally occurring crosslinking agent, which is less cytotoxic than the others[12] can be exploited to prepare crosslinked agarose-based materials for super absorbent and biomedical applications.

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