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Functional modification of agarose: A facile synthesis of a fluorescent agarose-tryptophan based hydrogel

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ABSTRACT

Microwave assisted facile synthesis of a fluorescent agarose-L-tryptophan hydrogel material employing carbodiimide chemistry (dicyclohexylcarbodiimide/4-dimethylaminopyridine; DCC/DMAP) has been described. The product formed fluorescent hydrogel at 1–1.5% (w/v), exhibiting fluorescence emission in water (λ_{max} 350 nm; 1×10^{-4} M), which was significantly higher (ca. 65%) than that of tryptophan at the same concentration. Subsequently, the agarose ester was cross linked with the natural cross linker genipin to yield a blue hydrogel (G-Ag-Trp_{Est}), confirming thereby the insertion of tryptophan moiety on to agarose backbone. Both the ester and cross linked hydrogels demonstrated gelling characteristics similar to agarose and were stable across a wide range of pH media (pHs 1.2, 7.0 and 12.5) under ambient conditions. These tryptophan containing fluorescent hydrogel materials may find applications in biomedical and pharmaceutical industries as potential radical scavengers and sensors.

1. Introduction

Hydrogels are three dimensional networks formed by many high or low molecular weight molecules in water and are being considered as a very good format for various applications including tissue engineering, vehicles for controlled drug release etc. (Hardy, Hirst, Ashworth, Brennan, & Smith, 2007; Hirst, Escuder, Miravet, & Smith, 2008; Lee & Mooney, 2001; Naskar, Palui, & Banerjee, 2009; Soussan, Cassel, Blanzat, & Rico-Lattes, 2009). After the advent of the concept of exciting green fluorescent protein (GFP) (Weiss, 2008). It is felt that study of biologically marked technologies and fluorescent materials prepared from biomacromolecules having excellent biocompatibility would be worthwhile. Reports are available of using biomacromolecules such as amino acids, carbohydrates for the preparation of fluorescent hydrogels with tailor-made properties (Bhuniya & Kim, 2006; Roy & Banerjee, 2011; Wu, 2008).

Agarose, the red seaweed polysaccharide is widely used in biomedical and bioengineering applications. The basic disaccharide repeating units of agarose consists of (1,3) linked β-D-galactose (G) and (1,4) linked α-L-3,6-anhydrogalactose (A) (Fig. 1a) (Araki, 1966).The aromatic amino acid L-tryptophan is widely used in human nutrition, pharmaceuticals and food industries (Fig. 1b). Genipin (Fig. 1c) is a naturally occurring compound extracted from the *Gardenia* fruit, which is widely used in herbal medicine, and recently it has emerged as a natural cross linking agent for different polymeric materials containing free amino groups (Akao, Kobashi, & Aburada, 1994; Djerassi, Gray, & Kincl, 1960; Touyama et al., 1994).

Microwave assisted esterification is one of the facile ways for modifying polysaccharides. This technique has been used in the synthesis of polysaccharide based materials (Biswas, Shogren, Kim, & Willett, 2006; Biswas et al., 2007, 2008). Synthesis of polysaccharide ester conjugates using *N,N′*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as activating agents for carboxylic acid have been studied widely (Heinze, Liebert, & Koschella, 2006; Kalaskar et al., 2010; Kapus'niak, Ciesielski, Kozioł, & T, 1999).

Amino acids containing synthetic polymers have assumed significant prominence as a new class of bioactive polymeric materials (Vriezema et al., 2004). Recently, Siepert et al. (2012) described the application of tryptophan, a 3-substituted indolic amino acid, in short-chain fluorescent tags for on-line detection of functional recombinant proteins. Tryptophan derivatives have also been reported to be free radical scavengers and anitoxidants (Peyrot & Ducrocq, 2008; Reiter, Tan, Cabrera, & D'Arpa, 1999). In a continued programme of functional modification of agarose in our laboratory, employing substrate molecules of biological significance, we have recently reported the syntheses of fluorescent derivatives of agarose and carrageenan by reacting with guanine, adenine, and cytosine via grafting techniques (Oza, Meena, Prasad, Paul, & Siddhanta, 2010; Oza, Meena, & Siddhanta, 2012; Oza, Prasad, & Siddhanta, 2012). In this article we report the synthesis of a new

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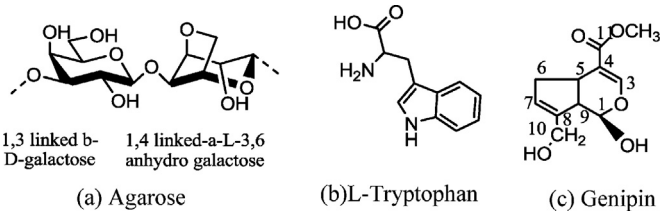


Fig. 1. Chemical structures (a) repeating unit of agarose (b) L-tryptophan (c) genipin.

fluorescent agarose-L-tryptophan based hydrogel material and its genipin cross linked blue hydrogel.

2. Experimental

2.1. Materials and instruments

Agarose was extracted from the red seaweed *Gracilaria dura* of Indian waters following the method reported in the literature (Meena, Prasad, & Siddhanta, 2007). The gel strength, sulphate and ash contents of the agarose were 950 g cm^{-2} (0.5% gel), ≤0.25% and 0.9% respectively. Fmoc-L-tryptophan (Fmoc-Trp) was purchased from M/s. Spectrochem Chemicals Ltd., Mumbai, India. L-Tryptophan was purchased from Biogen Chemicals and Pharmaceuticals Ltd., New Delhi. The naturally occurring cross linker genipin was purchased from M/s. Challenge Bioproducts Co. Ltd., Taiwan. Milestone Start-S (Italy) programmable microwave reactor (Model Start-S; Terminal T260; Line Voltage 230 V; Magnetron S.N. 131528; Frequency 2450 Hz) was used for the reaction. The characterizations were done by FT-IR analysis using a Perkin-Elmer FT-IR machine (Spectrum GX, USA) on a KBr disc (2 mg sample in 600 mg KBr). ^{13}C NMR spectra were recorded on a Bruker Avance-II 500 (Ultra shield) Spectrometer, Switzerland, at 125 MHz. Samples (agarose, Ag-Trp$_{Est}$) were dissolved in D_2O (60 mg mL^{-1}) and spectra were recorded at 70 °C, using DMSO as internal standard (ca. δ 39.5 ppm) (Rees, 1969). The ^{13}C NMR spectrum of L-tryptophan was recorded at ambient temperature. The ^{1}H NMR spectra of agarose, genipin and derivatives (Ag-Trp$_{Est}$, G-Ag-Trp$_{Est}$) were recorded (10 mg mL^{-1} of D_2O) at ambient temperature.

The UV–vis absorption spectra of the modified and non-modified agarose (1×10^{-4} M in H_2O) were recorded on a Varian CARY 500 UV–VIS-NIR Spectrophotometer, Pittsburgh, USA. The fluorescence spectra were recorded at room temperature on a Perkin-Elmer Spectrofluorimeter LS-50B, USA. The fluorescence emission spectra of agarose, Ag-Trp$_{Est}$, and G-Ag-Trp$_{Est}$ were measured at a concentration 1×10^{-4} M in distilled water as well as of tryptophan solution at 1×10^{-4} M, the molar equivalents present in the products respectively, using excitation and emission slits 1.0/1.0 nm. Tryptophan, Ag-Trp$_{Est}$ and G-Ag-Trp$_{Est}$ were excited at 280 nm with an emission at 340 nm.

The molecular weights (Mn, Mw) and polydispersity index (PDI) were measured by gel permeation chromatography (GPC) on a Waters Alliance 2695 machine with RI 2414 detector. Columns (Ultra hydragel 120, Ultra hydragel 500) were eluted with 0.1 M $NaNO_3$ at a flow rate of 0.5 mL min^{-1}. Oven and flow cell temperatures were maintained at 45 °C for all measurements. Dextrans of different molecular weights, e.g. 4.4×10^3, 4.3×10^4, 1.96×10^5, and 4.01×10^5 Da were used as standards for calibration.

Gel strengths of agarose, Ag-Trp$_{Est}$ (DS$_{Est}$ 0.70) and G-Ag-Trp$_{Est}$ were measured in 0.5% (w/v) gel at 20 °C on a Nikkansui-type gel tester (Kiya Seisakusho Ltd., Japan). The gelling and melting temperatures of gel samples were recorded as reported earlier (Mehta, Meena, Prasad, Ganesan, & Siddhanta, 2010). For measurement of gelling temperature, 10 mL (1.5%, w/v, 90 °C) hot sol of sample was allowed to cool gradually and a thermometer was emerged in the sol. The temperature, at which the thermometer left a mark on to the gel, after its withdrawal, was recorded. For melting temperature, the gel was heated on a water bath and one iron ball (weight ca. 1.0 g) was placed on the surface of the gel. The temperature at which the ball touched the bottom of the tube was recorded. Apparent viscosity was measured (in 0.5%, w/v sample) on a Brookfield viscometer (DV-II $^{+}$Pro), using SC4-18 spindle at 60 rpm and 80 °C.

2.2. Synthesis of Ag-Trp$_{Est}$

In a typical batch, the dried agarose (307 mg, 1.0 mmol w.r. to repeating unit of the polymer) was dissolved in N,N-dimethylformamide (DMF) at 80 °C for 3 min in a microwave reactor under stirring. Molar ratio of the agarose polymer was calculated on the basis of its repeating unit. To the solution, a pre-solubilized mixture of Fmoc-L-tryptophan (427 mg, 1.0 mmol), DCC (808 mg, 4.0 mmol), and DMAP (30.5 mg, 0.25 mmol) in DMF were added and the reaction was carried out under microwave irradiation at 80 °C for 20 min applying 400 W power. The reaction temperature and time were optimized on the basis of the highest yield of the ester. After the completion of reaction, the product was isolated by precipitation using isopropyl alcohol (reaction mixture-IPA, 1:2, v/v). The precipitated product was washed with IPA (10 mL × 4.20 min each) under stirring to remove excess of the reagents followed by drying under vacuum. The dried product was treated with 10% piperidine solution in DMF (10 mL) for 2 h under stirring at ambient conditions for removing the protecting group Fmoc. Finally, the product was isolated by precipitation with IPA followed by washing and drying as described above. The product formed hydrogel at 1–1.5% (w/v).

2.3. Cross linking of Ag-Trp$_{Est}$ (DS$_{Est}$ 0.70)

The cross linking reaction of Ag-Trp$_{Est}$ (DS$_{Est}$ 0.70) with genipin was carried out following the method reported earlier (Meena et al., 2007b). The Ag-Trp$_{Est}$ (DS$_{Est}$ 0.70, 100 mg) was dissolved in 10 ml distilled water at 120 °C for 20 min using an autoclave followed by the addition of genipin 1% (w/v) solution in methanolic water. The reaction mixture was allowed to stand for 48 h under ambient conditions, which formed a gel on cooling. The reaction mixture started assuming light blue colour after 120 min, the blue colour intensified gradually with the passage of time. The gelled reaction mixture was worked up to obtain the genipin cross linked Ag-Trp$_{Est}$ hydrogel by dewatering the gel with IPA (1:2, v/v) for 24 h, followed by air-drying at 50 °C for 2 h.

2.4. Gelation degree

The gelation degree (G) of Ag-Trp$_{Est}$ and G-Ag-Trp$_{Est}$ hydrogels was calculated adopting the method reported in the literature (Meena, Prasad, & Siddhanta, 2009), using Eq. (1).

$$G = \frac{m_d}{m_{iso}} \quad (1)$$

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

A known weight of the dried Ag-Trp$_{Est}$ or G-Ag-Trp$_{Est}$ hydrogel was taken, which was swelled in different pH media (e.g., pHs 1.2, 7.0 and 12.5) for 10 h in separate experiments. The weight of the isolated gel samples were determined (m_{iso}). The isolated gel samples were dewatered with a hundred-fold excess of IPA overnight, washed with IPA carefully and dried at room temperature under reduced pressure, and the sample was weighed again (m_d). All the measurements were repeated three times and mean values were considered.

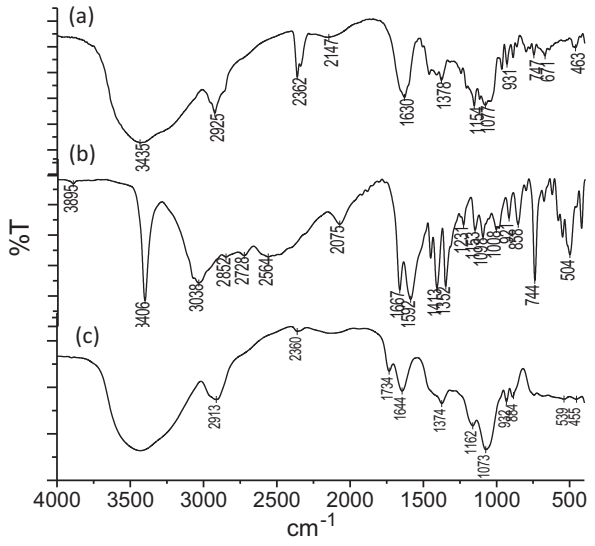


Fig. 2. FT-IR spectra of (a) tryptophan (b) Ag-Trp$_{Est}$.

2.5. Equilibrium swelling (ES)

The equilibrium swelling of agarose, Ag-Trp$_{Est}$ and G-Ag-Trp$_{Est}$ dried hydrogels in aqueous media having different pHs e.g. 1.2, 7.0 and 12.5 were measured in this study. In the swelling measurements, the dry weighed hydrogel was immersed in aqueous media having different pHs separately. After the desired soaking time had elapsed, the wet samples were wiped with filter paper to remove excess liquid, and weighed (W_t). In this study, the W_t had become constant after the 1 h of soaking in all pH media. The hydrogel of the products Ag-Trp$_{Est}$ and G-Ag-Trp$_{Est}$ remained stable more than a week in all pH media, while agarose hydrogel started losing weight in acidic medium after 24 h, slowly getting dispersed in the medium. Equilibrium swelling (ES) was calculated using Eq. (2) (Meena et al., 2007b).

$$ES = \frac{W_t - W_0}{W_0} \quad (2)$$

2.6. Characterization

Characteristic IR bands of agarose: ν_{max} (cm^{-1}) 3434*br* (O—H, stretching vibration), 2924*w* (C—H, stretching vibration), 1630*w* (H—O—H, stretching vibration of bound water), 1461*w* (CH_2, bending vibration), 1244*w* [S(=O)$_2$], 1154, 1077*br* (C—O—C, bending vibration of glycosidic linkage), 930 *m* (3,6-anhydro galactose), [band intensities: *br* = broad; *m* = medium; *w* = weak] (Christiaen & Bodard, 1983) (Fig. 2a).

Characteristic IR bands of L*-tryptophan*: ν_{max} (cm^{-1}) 3405s (N—H, stretching vibration), 3038*br* (C—H, stretching vibration), 2074*w* (C=C, stretching vibration), 1665s (—NH_3^+ deformation), 1591*br* (C=O, anti-symmetrical stretching vibration), 1454*w* (—COO^-, stretching vibration), 1412 (—C=O, symmetric stretching vibration), 1352*m* (—OH bending vibration), 1230*w* (CH_2, wagging vibration), 1152*m* (C—C, stretching vibration), 1098*m* (aryl group), 1007*m* (C—N, stretching vibration), 848*s* (substituted ring 1,4 distribution), 745 s (CH_2, rocking vibration), 696*br* (benzene ring), [band intensities: *br* = broad; *m* = medium; *s* = sharp; *w* = weak] (Çakir & Bicer, 2010) (Fig. 2b).

Characteristic IR bands of Ag-Trp$_{Est}$: ν_{max} (cm^{-1}) 3314*br* (—OH, stretching vibration), 3172*w* (—CH_2, stretching vibration), 1735*s* (—C=O, stretching vibration of ester), 1644*w* (H—O—H, stretching vibration of bound water), 1402*w* (C—C, bending vibration), 1377*w* (methylene group), 1255*w* (covalent sulphate group), 1119*br* (C—O—C, stretching vibration of glycosidic linkage), 932*m* (3,6-anhydro galactose), 892s (1,4-substituted ring), 749*w* (—CH_2, rocking vibration), 654*w*,*br* (benzene ring) [band intensities: *br* = broad; *m* = medium; *s* = sharp; *w* = weak] (Fig. 2c).

^{13}C NMR of agarose (125 MHz, D_2O, DMSO-d_6 as internal standard): δ 103.1 [C-1′], 98.9 [C-1], 82.9 [C-3′], 80.8 [C-3], 78.0 [C-4], 76.3 [C-5], 76.08 [C-5′], 71.0 [C-2′], 70.6 [C-2], 70.0 [C-6], 69.5 [C-4′], 62.1 [C-6′] (Fig. 3). These values were in good agreement with those reported in our previous work. (Rees, 1969).

^{13}C NMR of L*-tryptophan (125 MHz, D_2O, DMSO-d_6 as internal standard)*: δ 174.3 [C-a], 137.5 [C-j], 128.1 [C-e], 125.8 [C-k], 123.1 [C-h], 120.6 [C-f], 119.6 [C-g], 113.9 [C-i], 109.0 [C-d], 56.0 [C-b], 27.2 [C-c] (Fig. 3).

^{13}C NMR of Ag-Try$_{Est}$ (125 MHz, D_2O, DMSO-d_6 as internal standard): δ 170.7 [C-a], 136.1 [C-j], 127.5 [C-e], 124.9 [C-k], 123.2 [C-h], 121.1 [C-f], 118.5 [C-g], 111.5 [C-i], 109.7 [C-d], 101.5 [C-1′], 97.2 [C-1], 78.7 [C-3′, C-3, C-4], 73.5 [C-5, C-5′], 68.6 [C-2′, C-4′, C-2, C-6], 63.2 [C-6′ linked with L-Tryptophan], 60.2 [C-6′ linked with —OH], 55.2 [C-b], 30.5 [C-c] (Fig. 3).

2.7. Total nitrogen and degree of substitution

Total nitrogen was estimated by Kjeldahl method on a KEL PLUS-KES 201 Digestion unit attached to a KEL PLUS-CLASSIC DX Distillation unit (M/s PELICAN equipments, Chennai, India). The degree of substitution of L-tryptophan on agarose backbone was calculated on the basis of nitrogen contents of products, hence, it may be noted that isolated agarose did not contain nitrogen.

3. Result and discussion

3.1. Formation of Ag-Trp$_{Est}$ and G-Ag-Trp$_{Est}$

Optimization studies revealed that microwave irradiation for 20 min at 80 °C led to the formation of ester bond between carboxy terminal of Fmoc-L-tryptophan and hydroxyl groups of agarose in good yield (Scheme 1). The ester derivative of L-tryptophan with agarose having different molar ratios 1:0.5, 1:1, 1:2 was obtained in 92%, 87%, 83% yields. The respective degrees of substitution (DS$_{Est}$) and total nitrogen contents were 0.46, 0.70, 1.42 and 2.61 ± 0.1%, 3.90 ± 0.1%, 8.04 ± 0.1% (Table 1). The weight average molecular mass (Mw), number average molecular mass (Mn) and polydispersity index (PDI) of agarose and Ag-Trp$_{Est}$ (DS$_{Est}$ 0.70) are shown in (Table 1). The Mw and PDI data of Ag-Trp$_{Est}$ indicated that the agarose biopolymer backbone remained largely intact during the esterification by microwave irradiation. The carboxyl terminus of the Fmoc-L-tryptophan was engaged in the ester formation leaving behind a free amino group after the removal of Fmoc protecting group with 10% piperidine in DMF (Kalaskar et al., 2010). The amino group later participated in the genipin cross linking reaction and produced the cross linked product, the reaction mixture turned blue colour in 2 h (Scheme 1). The reaction possibly followed the similar mechanism as described in the literature (Scheme S1) (Chhatbar, Meena, Prasad, Chejara, & Siddhanta, 2011; Touyama et al., 1994).

3.2. Physicochemical properties

At the DS$_{Est}$ 1.42, the agarose ester (Ag-Trp$_{Est}$) became insoluble in water but was soluble in DMSO and DMF at 80 °C with difficulty, apparently due to the greater degree of hydrophobization of agarose backbone, while the product having DS$_{Est}$ 0.46, 0.70 were found to be readily soluble in water at 80–90 °C (Table 1). The water soluble product with greater DS$_{Est}$ (0.70) was allowed to react with genipin to produce the cross linked hydrogel. The

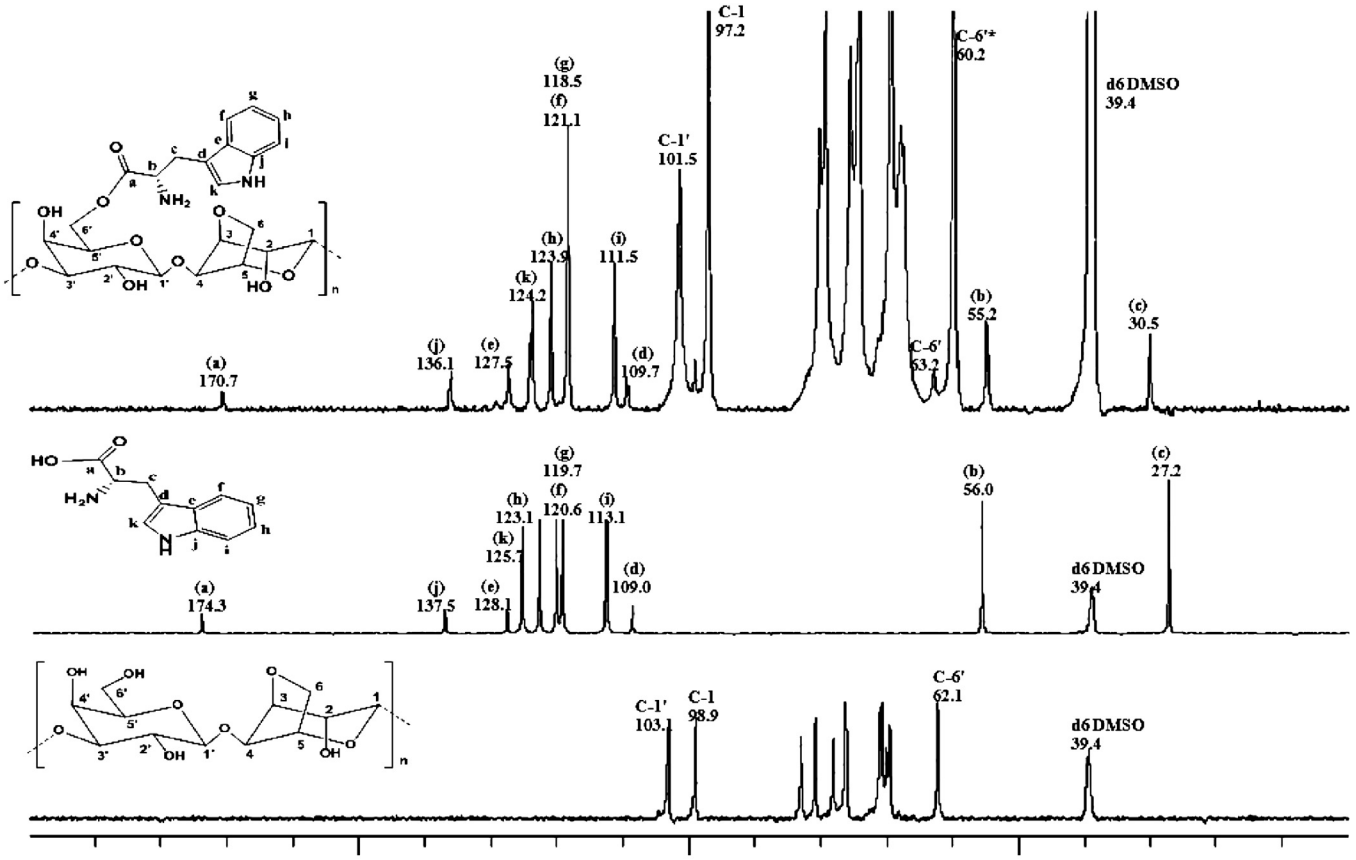
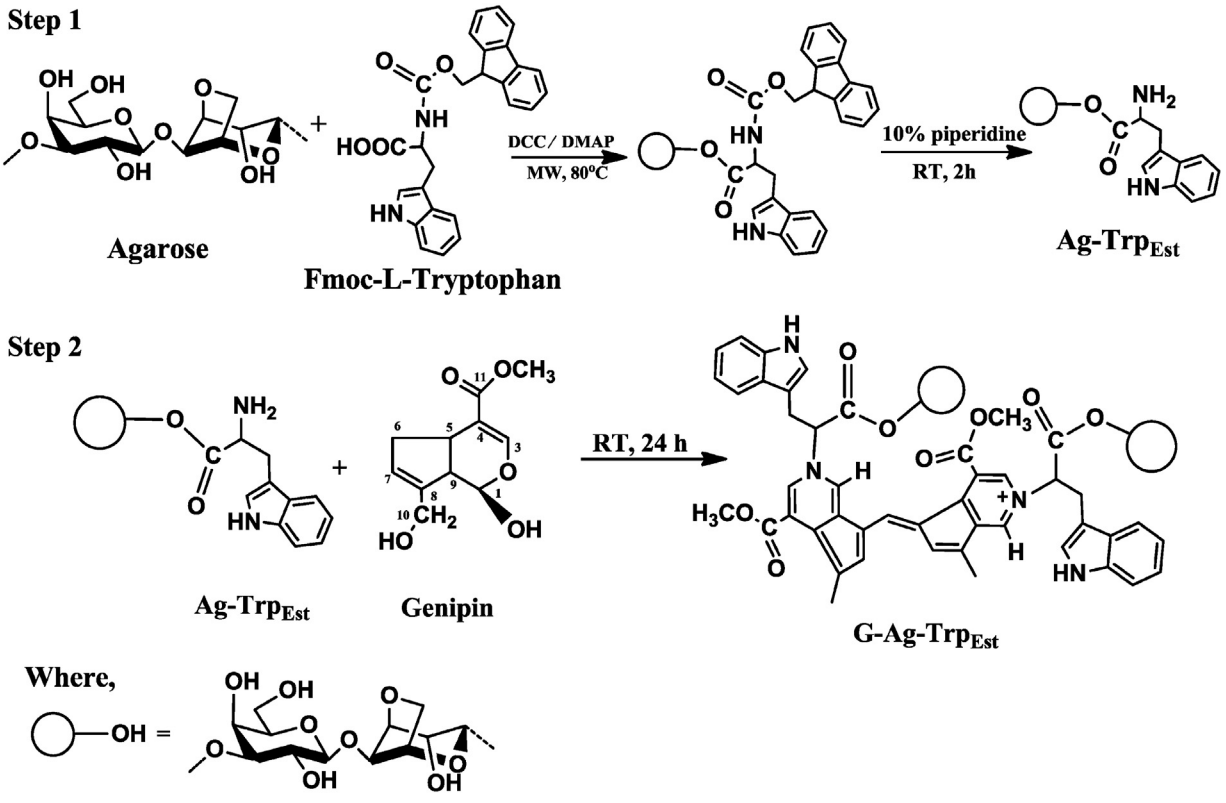


Fig. 3. ^{13}C NMR spectra of agarose, L-tryptophan, Ag-Trp_{Est} in D_2O (60 mg mL^{-1}) at 70 °C on 125 MHz spectrometer, d_6-DMSO was used as internal standard.



Scheme 1. Synthesis of hydrogel forming genipin cross linked agarose ester hydrogel.

Table 1
Yield (%) and physico-chemical properties of agarose and agarose ester derivatives.

Samples	Ag-Fmoc-Trp ratio	Isolated Yield (%)	Degree of esterification (DS_{Est})	Nitrogen contents (%)	Molecular weights (KDa)		Poly dispersity Index (PDI)	Solubility[a]
					Mw	Mn		
Agarose	NA	NA	NA	0%	117.571	44.570	2.638	Soluble[b]
Ag-Trp_{Est}	1:0.5	92	0.46	2.61 ± 0.1%,	127.117	38.767	2.836	Soluble[b]
Ag-Trp_{Est}	1:1	87	0.70	3.90 ± 0.1%,	172.015 (171.862)[c]	58.667(70.203)[c]	2.935(2.448)[c]	Soluble[b]
Ag-Trp_{Est}	1:2	83	1.42	8.04 ± 0.1%	NM	NM	NM	Soluble[d,e]
G-Ag-Trp_{Est}(DS_{Est} 0.70)	1:1	80	NA	3.19 ± 0.1%	140.735(137.225)[c]	78.163(66.235)[c]	1.802(2.071)[c]	Soluble[b]

NA: not applicable; NM: not measured.
[a] Soluble in respective solvents at 80–90 °C.
[b] Water.
[c] Values in the brackets are of parent product after swelling in pH 1.2 for 24 h.
[d] Dimethyl formamide (DMF).
[e] Dimethyl sulphoxide (DMSO).

physicochemical properties of Ag-Trp_{Est} (DS_{Est} 0.70) and those of the cross linked product (G-Ag-Trp_{Est}) were studied. The gel strength, gelling and melting temperature and apparent viscosities (80 °C) of Ag-Trp_{Est} (DS_{Est} 0.70) and the cross linked product (G-Ag-Trp_{Est}) were 970 ± 20 g cm^{-2}; 34 ± 1°, 98 ± 1°; 9.1 ± 1 cP and 930 ± 20; 34 ± 0.5°, 98 ± 0.5°; and 8.4 ± 1 cP, respectively. No significant variations were observed in these parameters of these two derivatives when compared with the parent polysaccharide agarose (Table 2). Both the products Ag-Trp_{Est} (DS_{Est} 0.70) and G-Ag-Trp_{Est} exhibited strongest gelling behaviour like agarose, which may be due to the limited presence of -*O*-tryptophan group on the agarose backbone facilitating formation of hydrogel networks through hydrogen bonding as well as increased occurrence of "junction zones" in the network, manifesting thereby the dominance of the agarose gel network. (Rees, 1969).

3.3. Swelling and stability of hydrogels in different pH media

In pH media (pHs 7.0 and 12.4), the gelation degrees (G) of the products [Ag-Trp_{Est} (DS_{Est} 0.70) and G-Ag-Trp_{Est}] were greater than those of agarose, while in pH media (pH 1.2) gelation degrees (G) decreased (Table 2). Equilibrium swelling (ES) values in pH 1.2 had the sequence Ag-Trp_{Est} > G-Ag-Trp_{Est} > agarose while in pHs 7.0 and 12.5 these had a sequence G-Ag-Trp_{Est} < Ag-Trp_{Est} < agarose. These may be due to the presence and absence of basic ionic character of the agarose ester *vis a vis* the neutral agarose polymeric chain (Mehta, Kondaveeti, & Siddhanta, 2011) (Figs. S2–S4). Swelling of agarose was the greatest in the pHs 7.0 and 12.5 in comparison to those of products. After 24 h the agarose gel at pH 1.2 started dispersing because of acid lability of agarose. On the other hand, the products Ag-Trp_{Est} (DS_{Est} 0.70) and G-Ag-Trp_{Est} were found to be stable in three pH media, pHs 1.2, 7.0 and 12.5 for more than a week (Figs. S2–S4). The swelling of Ag-Trp_{Est} (DS_{Est} 0.70) was relatively greater than G-Ag-Trp_{Est} at pHs 1.2, 7.0 and 12.5 presumably because of preferential protonation of the available free —NH_2 moieties facilitating intensive solvation with water molecules, leading to a stable ionic hydrogel. The genipin cross-linked product G-Ag-Trp_{Est} on the other hand, swelled comparatively less in acidic pH media since the —NH_2 group was engaged in the cross-linking reaction resulting in a structure restricting transport of water molecules inside the hydrogel network. In other words, reduced solvation in the absence of the ionic character of the cross-linked molecule (De et al., 2002; Mehta et al., 2011). The experiments on swelling were repeated with Ag-Trp_{Est}, and G-Ag-Trp_{Est} in pH 1.2 for 24 h, followed up by GPC experiment. It was found that the MW of the product decreased slightly in acidic pH after 24 h (Table 1). This indicated that no significant changes took place on the structure of the polysaccharides during the swelling experiments in acidic media, which is detrimental to the parent agarose polymer.

3.4. Spectral characterization of Ag-Trp_{Est}

Agarose derivatives Ag-Trp_{Est} have been synthesized with different degree of substitution shown in Table 1. The agarose derivative Ag-Trp_{Est} (DS 0.70) and G-Ag-Trp_{Est} (DS 0.70) were selected for characterization. The Ag-Trp_{Est} was characterized by FT-IR and ^{13}C NMR spectrometry. Formation of ester bond as a result of the reaction between hydroxyl group of agarose and carboxy termini of L-tryptophan was confirmed by the appearance of the sharp band at 1734 cm^{-1} in the FT-IR spectrum of Ag-Trp_{Est} (Fig. 2). The main characteristic absorption bands of agarose (3430, 1645, 1160, 1072 and 931 cm^{-1}) appeared in the FT-IR spectrum of Ag-Trp_{Est} indicating that the backbone of agarose remained intact during esterification. The ^{13}C NMR spectra of Ag-Trp_{Est} exhibited a peak at 170.7 ppm, confirming the presence of ester carbonyl group. The ^{13}C NMR spectra exhibit a signal at 30.5 ppm due to the methylene carbons of the tryptophan group and a signal at 55.2 ppm due to the C—N bond of the tryptophan group. The peaks between 60.2 and 101.5 ppm were due to the remaining methylene and methane carbons of agarose polymer moiety, the anomeric carbons appearing at 101.5 and 97.2 ppm. The peaks that appeared in the range 109.7–136.1 ppm can be assigned to the carbons of the aromatic ring of tryptophan (Fig. 3). The ^{1}H NMR spectra of the agarose polymer showed several signals between 3.3 and 5.1 ppm due to the protons linked to the sugar carbon atoms, however the peaks of agarose could not be seen clearly since these were

Table 2
Comparative physicochemical properties of agarose ester derivatives.

Products	Gel strength[a] (g cm^{-2})	Apparent viscosity (cP)[a]	Gelling temperature (°C)	Melting temperature (°C)	Gelation degree (G)		
					pH 1.2	pH 7.0	pH 12.5
Agarose	950 ± 20	8.2 ± 1	33 ± 0.5	98 ± 0.5	0.141	0.132	0.105
Ag-Trp_{Est} (DS_{Est} 0.70)	970 ± 20	9.1 ± 1	34 ± 0.5	98 ± 0.5	0.121	0.178	0.166
G-Ag-Trp_{Est} (DS_{Est} 0.70)	930 ± 20	8.4 ± 1	34 ± 0.5	98 ± 0.5	0.135	0.181	0.175

[a] Gel strength and apparent viscosity were measured in 0.5% (w/v) at 20 °C and 80 °C respectively.

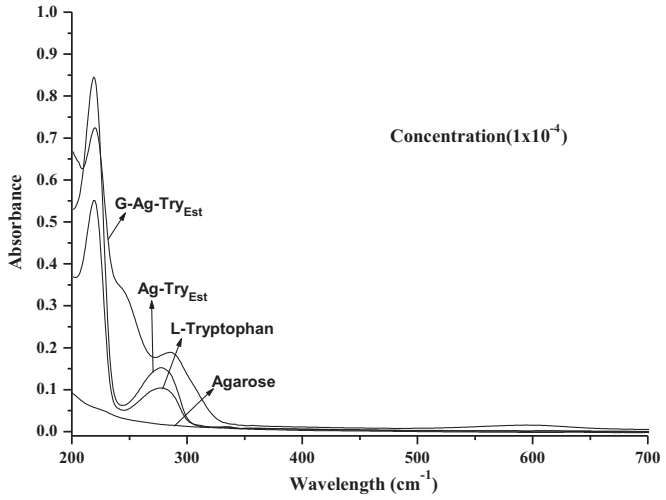


Fig. 4. UV–vis absorption spectra of agarose, L-tryptophan, Ag-Trp$_{Est}$ and G-Ag-Trp$_{Est}$ at Conc. 1×10^{-4} M in H_2O.

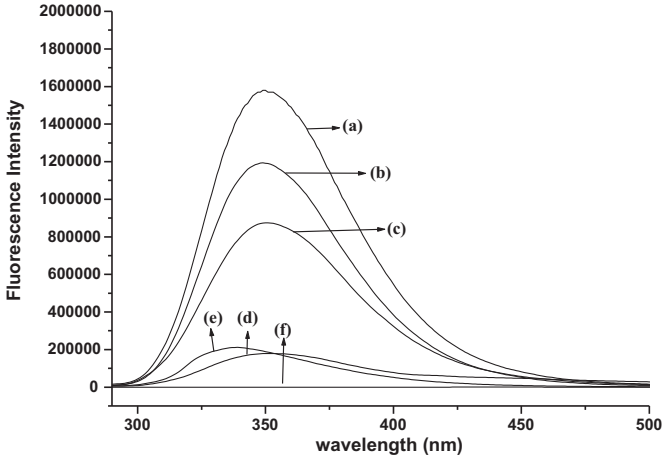


Fig. 5. Fluorescence emissions of (a) Ag-Trp$_{Est}$, 1×10^{-4} M; (b) L-tryptophan, 0.072 mg in 1×10^{-4} M (c) Ag-Trp$_{Est}$, 1×10^{-5} M; (d) Ag-Trp$_{Est}$, 1×10^{-6} M; (e) G-Ag-Trp$_{Est}$, 1×10^{-4} M (f) agarose, 5×10^{-5} M.

masked by the HOD of D_2O (Fig. S5a). The ^1H NMR spectra of the Ag-Trp$_{Est}$ showed several signals between 2.9 and 5.4 due to the protons linked to the sugar carbon atoms of agarose similarly the peaks appearing between 7.4 and 8.1 ppm due to the aromatic ring protons of tryptophan(Fig. S5c). The genipin cross linked product (G-Ag-Trp$_{Est}$) had a distinctive blue colour, which was further characterized by UV–vis spectrophotometry (Fig. 4) as well as by ^1H NMR spectrometry (Fig. S5a–e). The appearance of new proton signals that appeared in the range 2.08–2.79 ppm indicated that the genipin moiety was present in G-Ag-Trp$_{Est}$ (Fig. S5d).

3.5. UV–vis spectra

The non-modified agarose did not exhibit any absorption maxima in the UV–vis region, while L-tryptophan, genipin (Chhatbar et al., 2011; Touyama et al., 1994) and Ag-Trp$_{Est}$ exhibited absorption at λ_{max} (in distilled water, nm): 219 & 281 nm (ε 546 L mol^{-1} cm^{-1} & ε 104.6 L mol^{-1} cm^{-1}), λ_{max} 242 nm (ε 5000 L mol^{-1} cm^{-1}), λ_{max} 220 & 282 nm (ε 720.7 L mol^{-1} cm^{-1} ε 153.7 L mol^{-1} cm^{-1}) respectively. The genipin cross linked product G-Ag-Trp$_{Est}$ showed absorption bands at λ_{max} (in distilled water, nm): 241 nm (ε 342.8 L mol^{-1} cm^{-1}), 220 & 288 nm (ε 845.2 L mol^{-1} cm^{-1} & ε 193.2 L mol^{-1} cm^{-1}), 597 nm (17.2 L mol^{-1} cm^{-1}). The appearance of absorption maxima at λ_{max} 219 nm & 282 nm in Ag-Trp$_{Est}$ and at 241, 220, 288 and 597 nm in G-Ag-Trp$_{Est}$ indicated insertion L-tryptophan and subsequent cross linking of genipin involving the free amine group of Ag-Trp$_{Est}$, on the agarose backbone (Fig. 4). The new maxima at 597 nm had appeared due to the generation of an extended conjugation as a result of genipin cross linking manifesting the blue-colour (Scheme 1, Step-2). This is in good agreement with those reported in the literature (Chhatbar et al., 2011).

3.6. Fluorescence measurements

The fluorescence emissions (λ_{max} 350 nm) of pure tryptophan, agarose and the Ag-Trp$_{Est}$ derivative were measured at 1×10^{-4} M concentration (Fig. 5). Agarose at this concentration exhibited very low emissions, as previously observed (Oza et al., 2012a, 2012b). The emission spectrum of the modified agarose recorded in distilled water (1×10^{-4} M) solution exhibited emission maxima ($\lambda_{em,\,max}$) at 350 nm by excitation at 282 nm. The fluorescence emission of the agarose-tryptophan derivative in 1×10^{-4} M solution was significantly higher (ca. 65%) than that of tryptophan at the same concentration in aqueous solution, perhaps due to the hydration effect on tryptophan (Vivian & Callis, 2001). It is imperative that in addition to the hydration effect, certain molecular orientations in the tryptophan pendant agarose product perhaps precluded intermolecular electron transfer processes facilitating enhanced fluorescence (Oza et al., 2010, 2012a, 2012b)

4. Conclusions

A facile method has been described for the synthesis of fluorescent agarose-L-tryptophan based hydrogel. This was subsequently cross linked with the natural cross linker genipin to afford a blue hydrogel. These hydrogels exhibited similar strong gelling characteristics as those of agarose hydrogel, which were stable across the three pH media (pHs 1.2, 7.0 and 12.5) under ambient conditions than the parent agarose polysaccharide. Thus such modifications have imparted superior and new functional properties on agarose e.g. pH-stability and fluorogenicity, which may be of potential utility as hydrogels in biomedical and pharmaceutical domains as potential radical scavengers and as fluorescent probes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.04.034.

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