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Modification of agarose: 6-Aminoagarose mediated syntheses of fluorogenic pyridine carboxylic acid amides

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ABSTRACT

A facile 6-aminoagarose (AA) mediated synthesis of new fluorogenic amides of agarose with nicotinic (AA-NA) and picolinic acids (AA-PA) employing carbodiimide chemistry have been described. 6-Amino agarose (AA) was synthesized in a facile Mitsunobu-inspired microwave mediated method involving the reaction of agarose with phthalimide in presence of diisopropyl azodicarboxylate and triphenylphosphene (DIAD/TPP) followed by hydrazinolysis. All compounds were characterized by GPC, UV spectrophotometry, fluorescence spectroscopy, FT-IR, ^{1}H and ^{13}C NMR spectra. The fluorescence emissions (λ_{max} 430 and 412 nm) of 1×10^{-3} M solutions of AA-NA and AA-PA in water were significantly higher (ca. 82% and ca. 90%) than those of the molar equivalents (0.2 mg) of NA and PA present in the 1×10^{-3} M solutions of the amides, respectively. These fluorogenic pyridine carboxylic acid amides of agarose may find applications as sensors in biomedical and pharmaceutical industries.

1. Introduction

Fluorescent polysaccharides and their conjugates were prepared with an eye to identifying biomolecules; sensing pH as well as preparing cellulose based organic light emitting diode (Karakawa et al., 2007; Kobayashi, Urayama, & Ichishima, 1990; Qiu, Xu, Zhu, & Qiu, 2005; Schulz et al., 2009; Suizhou et al., 2003). Urreaga and De la Orden (2007), has reported modification of cellulose with amino compounds and their fluorescence properties. Chhatbar, Meena, Prasad, Chejara, and Siddhanta (2011), have reported the facile microwave-assisted method of synthesis of water-soluble fluorescent alginic acid amides employing four diamines as well as their crosslinked products. The synthesis of alginic acid amide conjugates using N,N-dicyclohexylcarbodiimide (DCC) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) as activating agents for the carboxylic acid group is widely studied and reported (Abu-Rabeah, Polyak, Ionescu, Cosnier, & Marks, 2005; Chiu, Lee, Chu, Chang, & Wang, 2008; Polyak, Geresh, & Marks, 2004). Johnston and co-workers have recently described a unique method of amide synthesis, harnessing reversed polarities of both the acid and amine moieties, which is totally different from the traditional approach based on the direct condensation of negatively charged amine and positively charged carbonyl of the carboxyl group. The process of formation of amide bonds is extremely important since this is the cornerstone of protein synthesis. Work is also going on to prepare polymeric fluorescent derivatives, which are essentially used as sensory materials, screening assays for enzymes, detection of peroxides, plastic colorants, electro-luminescence devices etc. (Cumming et al., 2001; Sanchez & Trogler, 2008; Wang, Huang, Xia, Gao, & Yan, 2002; Xia et al., 2004). Furthermore, water-soluble fluorescent polymers are particularly useful tracers for their potential to act as analogs in understanding the behavior of biological macromolecules (Gao, Wang, Yang, & Fu, 2004).

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) (Araki, 1966). Several methods for the synthesis of amino group containing polysaccharide derivatives i.e. 6-amino-6-deoxycellulose, amino cellulose esters and amino starch have been reported (Matsui, Ishikawa, Kamitakahara, Takano, & Nakatsubo, 2005; Pinto Zarth, Koschell, Pfeifer, Dorn, & Heinze, 2011; Dong et al., 2010). Cellulose of different degrees of polymerization was converted to amino group containing esters under homogeneous reaction conditions by ring-opening reaction of various lactams in the presence of p-toluene sulphonic acid chloride (TosCl) (Pinto Zarth et al., 2011). Synthesis

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of an aminopolysaccharide via polymerization of 6-amino-6-deoxy-D-glucose and 2,6-diamino-2,6-dideoxy-D-glucose reported by Kadokawa, Hino, Karasu, Tagaya, and Chiba (2000). The resulted aminopolysaccharide was expected to be useful in the development of glyco-technology (Kadokawa et al., 2000). Carbohydrates functionalized with groups e.g. —SH, —NH_2 and —COOH can be used as synthetic tools for the isolation and affinity-based purification of carbohydrate-binding proteins as well as determination of their structural specificity (Daniel, Eddie, Matthew, & Peter, 2004).

A very useful bioactive molecule is nicotinic acid (pyridine-3-carboxylic acid) or also known as niacin, vitamin B3, and vitamin PP, a well-known cholesterol-lowering agent, was selected for this investigation. Nicotinic acid, which occurs naturally in food when used as a medicine, can improve levels of blood fats (lipids) such as cholesterol and triglycerides (Carlson, 2005; Gille, Bodor, Ahmed, & Offermanns, 2008). Likewise, picolinic acid (pyridine-2-carboxylic acid), an isomer of nicotinic acid was also selected for this study. Picolinic acid is used as an intermediate to produce pharmaceuticals (especially local anesthetics) and its metal salts for the application of nutritional supplements. The acid is believed to form a complex with zinc that may facilitate the passage of zinc through the gastrointestinal wall and into the circulatory system. A study of rats found that dietary picolinic acid increases the turnover and excretion of zinc (Evans & Johnson, 1981). Picolinic acid is an endogenous metabolite of L-tryptophan (TRP) that has been reported to possess a wide range of neuroprotective, immunological, and anti-proliferative affects within the body (Grant, Coggan, & Smythe, 2009).

As part of a continuing program of functional modification of seaweed polysaccharides in our laboratory (Chhatbar, Godiya, & Siddhanta, 2012; Kondaveeti, Prasad & Siddhanta, 2013; Mehta, Kondaveeti, & Siddhanta, 2011; Oza, Meena, & Siddhanta, 2012), we report herein a facile 6-aminoagarose mediated synthesis of new fluorogenic amides of agarose with pyridine carboxylic acids viz. nicotinic and picolinic acids.

2. Materials and methods

Agarose was extracted from the red seaweed *Gracilaria dura* of Indian waters following the method reported in the literature (Meena et al., 2007). The gel strength, sulphate and ash contents of the agarose were 950 g cm^{-2} (0.5% gel), ≤0.25% and 0.9%. Other chemicals e.g. phthalimide, diisopropyl azodicarboxylate (DIAD), triphenyl phosphine (TPP), and hydrazine were purchased from M/s. Spectrochem Chemicals Ltd., Mumbai, India. Nicotinic acid (NA) and picolinic acids (PA) were purchased from M/s. SRL Chemicals Ltd., Mumbai, India. 1-Ethyl-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), 2-[N-morpholino]-ethanesulfonic acid (MES) buffer and N-hydroxysuccinimide (NHS) were purchased from M/s Sigma Aldrich, Mumbai, India. 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 reactions.

The characterizations were done by FT-IR analysis using a Perkin-Elmer FT-IR machine (Spectrum GX, USA) on a KBr disk (2.0 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 (Aminoagarose, AA-NA and AA-PA) were dissolved in D_2O (60 mg mL^{-1}) and spectra were recorded at 70 °C, d_6-DMSO was used as the internal standard (ca. δ 39.5 ppm) for AA-NA and AA-PA samples while it was d_3-acetonitrile for AA in the measurement of carbon-13 NMR spectra. A-Ph was dissolved in d_6-DMSO and the spectra were recorded at 70 °C and d_3-acetonitrile was used as internal standard. The ^{13}C NMR spectrum of nicotinic acid (NA) and picolinic acid (PA) were dissolved in d_6-DMSO (60 mg mL^{-1}) and spectra were recorded at ambient temperature. The ^1H NMR spectra of Agarose, AA, AA-NA and AA-PA derivatives were recorded (10 mg mL^{-1} of D_2O) at ambient temperature. The ^1H NMR spectra of Nicotinic acid and picolinic acid were recorded (10 mg mL^{-1} of d_6-DMSO) at ambient temperature.

The molecular weights (M_n, M_w) 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.

Differential scanning calorimetric (DSC) was measurements were done on a Mettler Toledo DSC822 equipment (Switzerland), using a rate 1 °C min^{-1} in the temperature range −10 °C to 110 °C. Gel strengths of agarose, A-Ph (DS 0.24) and G-AA (DS 0.20) 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 et al., 2011).

2.1. Scanning electron microscopy (SEM)

The changes in morphology of the different samples (AA-NA and Ag-PA) were determined using a scanning electron microscope (Carl-Zeiss), model LEO 1430 VP, Germany, at an accelerating voltage of 20 kV. A small amount of sample was mounted on aluminum stub and placed it in the vacuum chamber. All images were recorded at the same magnification.

2.2. Total nitrogen and degree of substitution (DS)

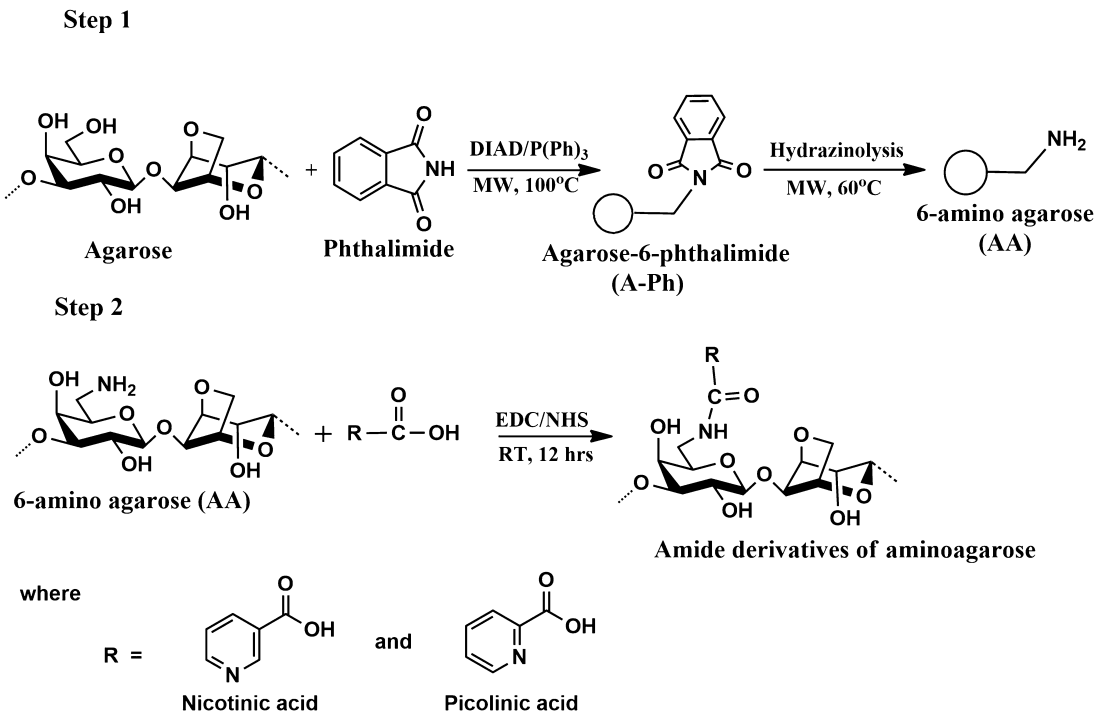
Total nitrogen was estimated by Kjeldahl method on a KEL PLUS-KES 201 Digestion unit attached to a KEL PLUS-CLASSIC DX Distillation unit (M/s PELICAN equipments, Chennai, India). The degree of substitution (DS) on agarose backbone was calculated on the basis of nitrogen contents of products, hence, it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

2.3. Synthesis of 6-amino agarose (AA)

In a typical batch, the dried agarose (306 mg, 1 mmol) was dissolved in N,N-dimethylformamide (DMF) in a microwave reactor (80 °C for 3 min). The agarose solution was cooled to 0 °C followed by the addition of phthalimide (73.56 mg, 0.5 mmol), triphenylphosphine (1049.16 mg, 4 mmol) and diisopropyl azodicarboxylate (787.57 μL, 4 mmol) and reaction was carried out under microwave irradiation at 100 °C for 15 min. The agarose phthalimide (A-Ph) derivative was isolated by precipitation with isopropyl alcohol (IPA) (1:2 v/v), washed with IPA and cold (10 °C) diethyl ether (10 mL × 4; 20 min of each) and dried in a vacuum desiccator at ambient temperature. A-Ph was subjected to hydrazinolysis with hydrazine hydrate (20 mmol) in 5.0 mL ethanol in a microwave reactor at 65 °C for 30 min (Scheme 1). The product (AA) was filtered and washed with hot (50 °C) ethanol (10 mL × 3) and dichloromethane (10 mL × 3) dried in a vacuum desiccators.

2.4. Synthesis of nicotinic acid (pyridine-3-carboxylic acid) and picolinic acid (pyridine-2-carboxylic acid) amide of agarose derivatives

0.036 g (0.5 mmol) of nicotinic acid was added to a 1% (w/v) solution of aminoagarose DS 0.20 (20 mL solution) in 0.1 M MES buffer,



Scheme 1. Formation of 6-amino agarose (AA) and amide formation with amino group of the aminoagarose.

pH 6.0. The reaction mixture was stirred at room temperature for 15 min to facilitate a homogeneous dispersion of the nicotinic acid in the reaction solution followed by the addition of 0.115 g (0.5 mmol) of NHS and 0.193 g (1 mmol) of EDC. After 12 h of stirring at room temperature the resulting mixture 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 to obtained final aminoagarose-nicotinic acid (AA-NA). Similarly the aminoagarose-picolinic acid (AA-PA) product was obtained followed by above method. The plausible mechanisms of formation of the amide products are shown in Scheme 1. The carboxyl terminus of the pyridine carboxylic acids was engaged in the amide formation with amino group of the aminoagarose.

2.5. Cross-linking of AA (DS 0.20)

The cross linking reaction of AA with genipin was carried out following the method reported earlier (Meena, Prasad, & Siddhanta, 2009). The AA (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 under ambient conditions, which formed a gel on cooling, for 24 h. 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 24 h. The gelled reaction mixture was worked-up to obtain the genipin cross linked AA hydrogel product (G-AA) by dewatering the gel with IPA (1:2 v/v) for 24 h, followed by air-drying at 50 °C for 2 h.

2.6. Gelation degree

The gelation degree (G) of Agarose, A-Ph (DS 0.24), AA (DS 0.20) and G-AA (DS 0.20) hydrogels was calculated adopting the method reported in the literature (Meena et al., 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 hydrogel was taken, which was swelled in different pH media (e.g., pH 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.

2.7. Equilibrium swelling (ES)

The equilibrium swelling of agarose, A-Ph (DS 0.24), AA (DS 0.20) and G-AA (DS 0.20) 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 hydrogel was weighed (W_0), which was immersed separately in aqueous different pH media. 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 1.0 h of soaking in all pH media. The hydrogel of the products remained stable more than a week in all pH media investigated, while agarose hydrogel started losing weight in acidic medium after 24 h. Equilibrium swelling (ES) was calculated using Eq. (2) (Meena et al., 2009).

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

2.8. UV–vis and fluorescence measurements

The UV–vis absorption spectra of the modified and non-modified agarose (1×10^{-3} 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 Aminoagarose, AA-NA and AA-PA were measured at a concentration 1×10^{-3} M in distilled water as well as of

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

Samples	Agarose-phthalimide ratio	Isolated yield (%)	Degree of substitution (DS)	Nitrogen contents (%)	Molecular weights (KDa)		Poly-dispersity Index (PDI)	Solubility[a]
					M_w	M_n		
Agarose	NA	NA	NA	0%	117.571	44.570	2.638	b,c,d
A-Ph	1:0.5	81.71	0.24	0.77 ± 0.1%	116.675	46.913	2.487	b,c,d
A-Ph	1:1	78.16	0.94	3.01 ± 0.1%	NM	NM	NM	c,d
A-Ph	1:2	72.69	0.90	2.88 ± 0.1%	NM	NM	NM	c,d
AA	1:0.5	76.00	0.20	0.91 ± 0.1%	116.215	49.417	2.351	b,c,d
AA	1:1	52.00	0.88	4.03 ± 0.1%	95.630	48.003	1.992	b,c,d
AA	1:2	53.33	0.82	3.76 ± 0.1%	54.512	12.833	4.247	b,c,d
G-AA	1:0.5	96.27	NA	0.89 ± 0.1%	115.812	52.285	2.215	b,c,d
AA-NA	1:0.5	85.25	0.20	NM	114.325	46.658	2.450	b,c,d
AA-PA	1:0.5	92.50	0.20	NM	113.965	45.269	2.517	b,c,d

NA, not applicable; NM, not measured.
[a] Soluble in respective solvents at 80 °C.
[b] Water ca. 7%.
[c] Dimethyl formamide (DMF) ca. 5% for agarose & 15% for other products.
[d] Dimethyl sulphoxide (DMSO) ca. 5% for agarose & 15% for other products.

nicotinic acid and picolinic acid solution at 1×10^{-3} M, the molar equivalents present in the products respectively, using excitation and emission slits 1.0/1.0 nm. Nicotinic acid and AA-NA were excited at 262 nm with an emission at 430 nm; picolinic acid and AA-PA were excited at 262 nm with an emission at 412 nm.

3. Result and discussion

3.1. Physicochemical properties of 6-amino agarose (AA) and pyridine carboxylic acid amides (AA-NA and AA-PA)

In an optimization study three 6-aminoagarose derivatives were synthesized, having DS 0.20, 0.82 and 0.88 (Table 1). These were obtained as a result of the reactions employing agarose and phthalimide in the following molar proportions – 1:0.5, 1:1 and 1:2, and subsequent hydrazinolysis. It was observed that higher the DS, lower was the swellability exhibiting a convincing trend. The one with DS 0.20 showed good swelling properties in all pH media (e.g. 1.2, 7.0 and 12.5), therefore it was employed for detailed study of its properties.

Optimization studies revealed that microwave irradiation for 15 min at 100 °C led to the functionalization of agarose by phthalimide (A-Ph) through the formation of C—N bond in good yield (Scheme 1). The reaction was carried out employing different agarose–phthalimide molar ratios i.e. 1:0.5, 1:1, 1:2 which yielded 81.71%, 78.16%, 72.69% of products respectively. The respective degrees of substitution (DS) and total nitrogen contents were 0.24, 0.94, 0.90 and 0.77 ± 0.1%, 3.07 ± 0.1%, 2.88 ± 0.1% (Table 1). At the DS 0.94, the A-Ph became insoluble in water but was readily soluble in DMSO and DMF, apparently due to the greater degree of hydrophobization of agarose backbone, while the product having DS 0.24 was found to be soluble in water at 80–90 °C (Table 1). Aminoagarose (DS 0.20) was soluble in water at 80 °C. It was found that higher the DS i.e. 0.88 in AA, having relatively lower M_w, exhibited gel strength <100 g cm^{-1} (Table 1). This was presumably due to the significant changes in the matrix structure with the insertion of greater degree of amino groups than those in AA with DS 0.20. The AA product having DS 0.20 was employed for cross-linking reaction to ascertain the insertion of —NH_2 group on to agarose. The amino group later participated in the genipin cross linking reaction and produced the cross linked product (G-AA), the reaction mixture turned dark blue from light blue in 24 h. This confirmed the introduction of a free -NH_2 group on to agarose (Fig. S1). The reaction possibly followed the similar scheme as described in the literature (Chhatbar et al., 2011). The cross linked product (G-AA) exhibited a similar gel strength (920 g cm^{-2}) to the parent AA gel (Table 2). All the three products A-Ph (DS 0.24), AA (DS 0.20) and G-AA (DS 0.20) exhibited comparable gel strength than that of agarose, which was presumably due to the low level of substitution on the hydroxyl groups of agarose backbone facilitating formation of hydrogel networks through hydrogen bonding facilitating enhanced "junction zones" in the network (Meena et al., 2007; Kondaveeti et al., 2013).

In all pH media (pH 1.2, 7.0 and 12.4), the gelation degrees (G) of the products [A-Ph (DS 0.24), AA (DS 0.20) and G-AA (DS 0.20)] were greater than those of agarose gel (Table 2). Equilibrium swelling (ES) values at pH 1.2 had the sequence agarose > AA (DS 0.20) > G-AA (DS 0.20) while at pH 7.0 and 12.5 it was agarose > G-AA (DS 0.20) > AA (DS 0.20). Swelling of agarose was the greatest in all pHs 1.2, 7.0 and 12.5 in comparison to those of products, but after 24 h the agarose gel at pH 1.2 started dispersing because of acid liability of agarose. On the other hand, the products AA (DS 0.20) and G-AA (DS 0.20) were found to be stable in three pH media, pH 1.2, 7.0 and 12.5 for more than 24 h (Figs. S2–S4). It may be noted that swelling of agarose hydrogel decreased in acidic pH after 24 h, which may be due to the direct consequence of acid liability of agarose polymer in comparison to those in pH 7.0 and 12.5 media. The experiments on swelling were repeated with AA, A-Ph and G-AA in pH 1.2 for 24 h, followed up by GPC experiments. It was found that the MW of the product decreased slightly (ca. 10%) in acidic pH after 24 h (Table

Table 2
Comparative physico-chemical properties of agarose derivatives.

Products	Gel strength[a] (g cm^{-2})	Gelling temp. (°C)	Melting temp. (°C)	Gelation degree[a] (G)		
				pH 1.2	pH 7.0	pH 12.5
Agarose	950 ± 20	33 ± 0.5	98 ± 0.5	0.141	0.132	0.105
A-Ph (DS ~0.24)	920 ± 20	34 ± 0.5	98 ± 0.5	0.354	0.334	0.316
AA (DS ~0.20)	900 ± 20	33 ± 0.5	98 ± 0.5	0.342	0.513	0.434
G-AA (DS ~0.20)	920 ± 20	33 ± 0.5	98 ± 0.5	0.322	0.471	0.416

[a] Gel strength and gelation degree were measured in 0.5% (w/v) gel at 20 °C in water.

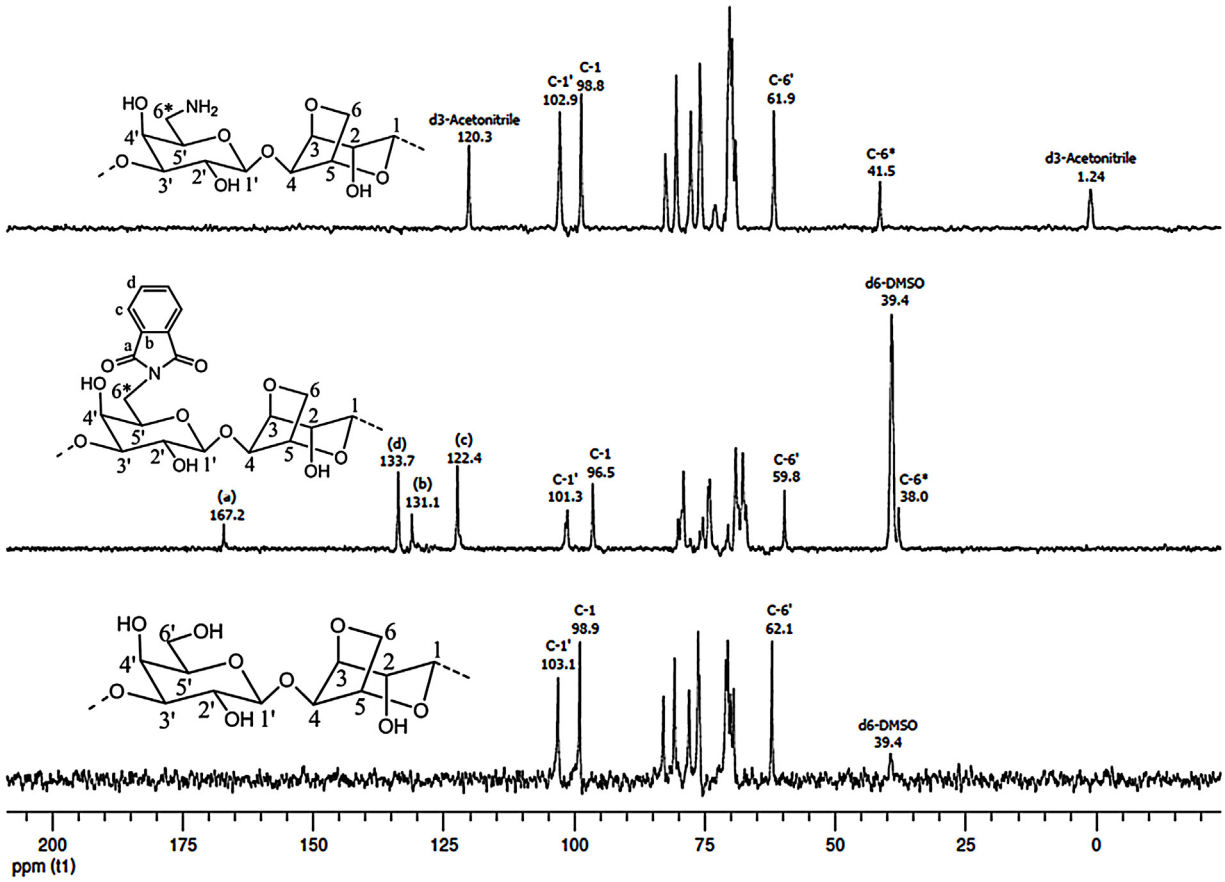


Fig. 1. ^{13}C NMR spectra of (a) AA (b) A-Ph and (c) agarose in D_2O (60 mg mL^{-1}) at 70 °C on 125 MHz spectrometer, d_6-DMSO was used as internal standard for A-Ph and agarose while CD_3CN for AA.

S5). This indicated that no significant changes took place on the structure of the polysaccharides during the swelling experiments in acidic media.

Further the studies revealed that for 12 h at ambient condition led to the formation of amide bond between carboxy terminal of pyridine carboxylic acids and amino groups of amino agarose (DS 0.20) (Scheme 1). Optimization studies revealed that 15 min duration for amide formation reaction was sufficient to produce the respective amines in good yield and purity. The yields of the amide derivatives namely AA-NA and AA-PA were 85.25% and 92.50% respectively (Table 1). The respective degrees of substitution (DS) were determined by UV spectroscopy, based on the extinction coefficient of the highest peak in the UV absorption spectra of the polymers concerned, λ_{max} 260 nm (ε 5000 L mol^{-1} cm^{-1}) (Nowakowska, Zapotoczny, Sterzel, & Kot, 2004). The degree of substitutions (DS) of AA-NA and AA-PA were 0.2 (Table 1). The amide derivatives AA-NA and AA-PA were water soluble at room temperature due to the limited presence of nicotinic and picolinic acids group on the aminoagarose backbone.

The weight average molecular mass (M_w), number average molecular mass (M_n) and polydispersity index (PDI) of agarose and functionalized products A-Ph (DS 0.24), AA (DS 0.20, 0.88, 0.82) are furnished in the Table 1. The M_w and PDI data of A-Ph (DS 0.24) and AA (DS 0.20) indicated that the agarose biopolymer backbone remained largely intact during the reactions by microwave irradiation, however, in case of other products, with increased molar ratio, M_w of the product decreased, apparently due to the depolymerization of the agarose biopolymer (Table 1). The M_w and PDI data of AA-NA and AA-PA indicated that the agarose biopolymer backbone remained largely intact during the reaction condition (Table 1).

3.2. Differential scanning calorimetric studies (DSC)

The amino agarose (AA) having DS = 0.20 formed strong hydrogel, like agarose, presumably due to the low but adequately optimum substitution of amino group for sustaining comparable hydrogel properties. On the basis of the dynamic scanning calorimetric (DSC) data of AA and G-AA, it can be concluded with reasonable degree of confidence that these were distinctly individual compounds and not the mixed ones. AA one single endothermic peak (T_g) in DSC (99 °C in the temperature range −10 °C to 110 °C) were obtained, indicating single gelation characteristic (Fig. S6). It may be noted here that the parent polysaccharide, further corroborating the formation of a new polysaccharide derivative (Prasad, Siddhanta, Rakshit, Bhattacharya, & Ghosh, 2005).

3.3. Spectral characterizations

3.3.1. FTIR spectroscopy

The A-Ph, AA, AA-NA and AA-PA were characterized by FTIR spectrometry. The agarose derivatives A-Ph (DS 0.24), AA (DS 0.20) and G-AA (DS 0.20) were selected for characterization. Functionalization of phthalimide on the agarose backbone via the substitution of hydroxyl groups was confirmed by the appearance of the new bands at 1728 (carbonyl) and 1554 (aromatic moiety) cm^{-1} in the FT-IR spectrum of A-Ph (Krishnakumar, Balachandran, Chithambarathanu, 2005). The conversion of A-Ph to AA through hydrazinolysis was also confirmed by the absence of these absorption bands in the FT-IR spectrum of AA (Fig. S7). Formation of amide bond as a result of the reaction between amino group of aminoagarose and carboxyl termini of picolinic acid (AA-PA) and nicotinic acid (AA-NA) were confirmed by the appearance of the

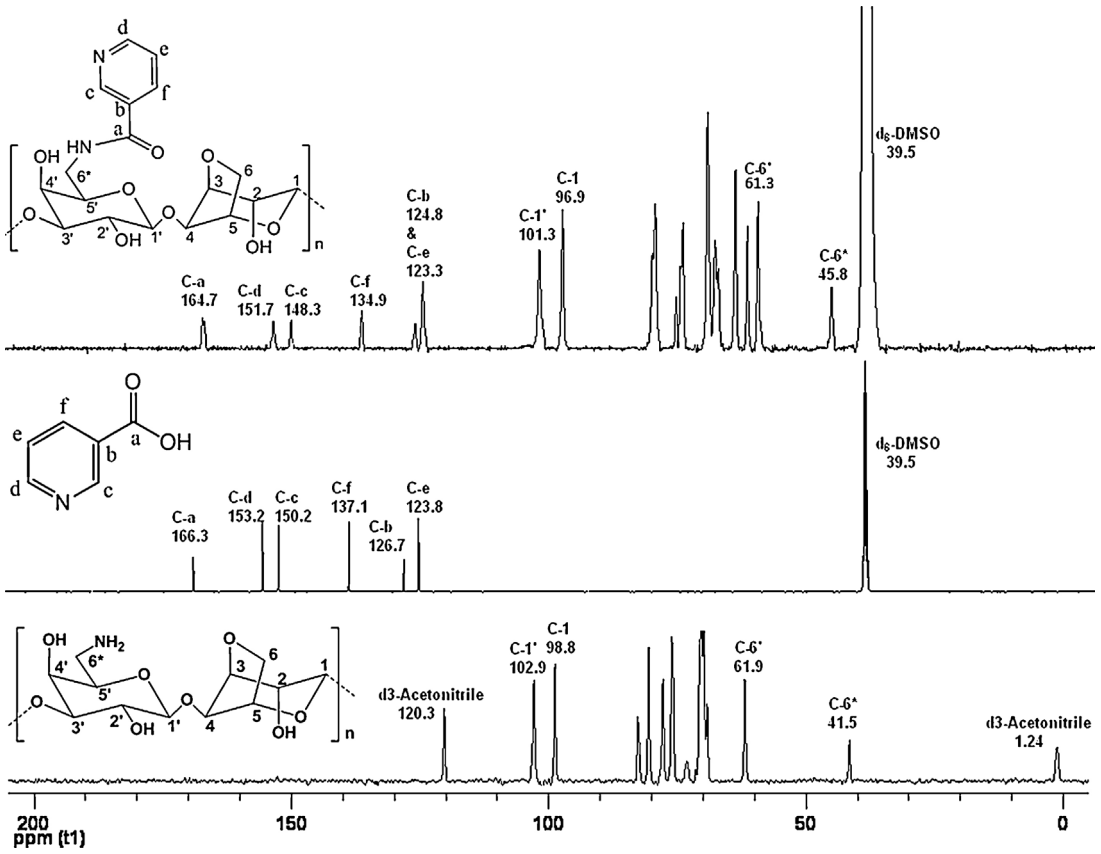


Fig. 2. ^{13}C NMR spectra of aminoagarose, nicotinic acid, AA-NA in D_2O (60 mg mL^{-1}) at ambient condition on 125 MHz spectrometer, d_3-acetonitrile and d_6-DMSO was used as internal standard.

bands at 1676 and 1698 cm^{-1} in the FT-IR spectra respectively. The intensity of band characteristic for a hydroxyl group at ~3405 cm^{-1} in AA-NA and AA-PA derivatives reduced in comparison to parent aminoagarose indicating the formation of amide bond between the amino group of aminoagarose and carboxyl group of nicotinic acid and picolinic acids. The characteristic absorption bands of aminoagarose were 3438, 1642, 1160, 1074 and 932 cm^{-1} indicating that the backbone of aminoagarose remained largely intact during the reaction (Fig. S8) (Atac, Karabacak, Karacaa, & Kosea, 2012).

3.3.2. ^{13}C NMR spectrometry

The A-Ph, AA, AA-NA and AA-PA were characterized by ^{13}C NMR spectrometry. The ^{13}C NMR spectra of A-Ph (DS 0.24) exhibited peaks at 167.2, 133.7, 131.1, 122.4, 38.0 ppm along with the peaks of agarose backbone structure, confirming the bond formation of phthalimide with agarose biopolymer (Fig. 1). The appearance of a new peak at 41.4 ppm in ^{13}C NMR spectra of AA confirmed the insertion of amino group at C-6 position on agarose backbone (Fig. 1) (Oza et al., 2012). The ^{13}C NMR spectra of aminoagarose, nicotinic acid, AA-NA, picolinic acid and AA-PA are shown in Figs. 2 and 3. The ^{13}C NMR spectra of AA-NA and AA-PA exhibited a peak at 164.7 ppm and 164.8 ppm confirming the presence of amide carbonyl group, which in nicotinic acid and picolinic acid appeared at 166.3 ppm and 166.1 ppm respectively. The anomeric carbons of AA-NA appeared at 101.4 ppm and 98.8 ppm and AA-PA appeared at 103.3 ppm and 96.4 ppm as opposed 102.9 and 98.9 ppm in aminoagarose, indicating insertion of nicotinic acid and picolinic acid on aminoagarose polymer backbone respectively. Further, the carbon resonance of the C—N bond of aminoagarose at 41.5 ppm exhibited a downfield shift to 45.8 ppm in AA-NA and 46.2 ppm in AA-PA, which clearly indicated that the amidation took place involving the C-6 amino group of aminoagarose. The peaks that appeared in the range 123.3–151.7 ppm were attributed to the aromatic carbons of the nicotinic acid in AA-NA and 127.3–148.6 ppm in AA-PA respectively (Figs. 2 and 3). The group of resonances for the methylene and methane carbons of AA-NA and AA-PA in the range 79.8–61.9 ppm showcased a different pattern than that of aminoagarose in the range 82.6–61.8 ppm, corroborating again the formation of new amide derivatives. The assignments of carbon resonances were done by comparison with literature values as well as with data obtained with ChemBioDraw Ultra 11.0 (Atac et al., 2012; Kondaveeti et al., 2013).

3.3.3. ^{1}H NMR spectrometry

The proton NMR spectra of agarose, AA, nicotinic acid, AA-NA, picolinic acid and, AA-PA were depicted in the Supplemental Data (Figs. S9 and S10). The ^{1}H NMR spectra of the aminoagarose polymer showed several signals between 3.14 and 5.31 ppm, as against the proton resonances of agarose signals between 3.33 and 5.14 ppm due to the protons linked to the sugar carbon atoms, however the peaks of agarose could not be seen clearly since these were masked by the *H*OD of D_2O (Fig. S9a and b). The ^{1}H NMR spectrum of AA-NA exhibited two sets of peaks of anomeric protons at 5.19–5.07 ppm and 4.81–4.63 ppm similarly AA-PA exhibited two sets of peaks of anomeric protons at 5.04–4.85 ppm and 4.69–4.57 ppm indicating a pervasive anisotropy in the molecular structure of AA-NA and AA-PA respectively (Fig. S10b and d), as against the two anomeric proton resonances of aminoagarose appearing at 5.31 and 5.18 ppm. The remaining signals in the range 4.51–2.80 ppm and 4.49–3.29 ppm were attributed to the methylene and methine protons of AA-NA and AA-PA respectively, while those of aminoagarose appeared in the range

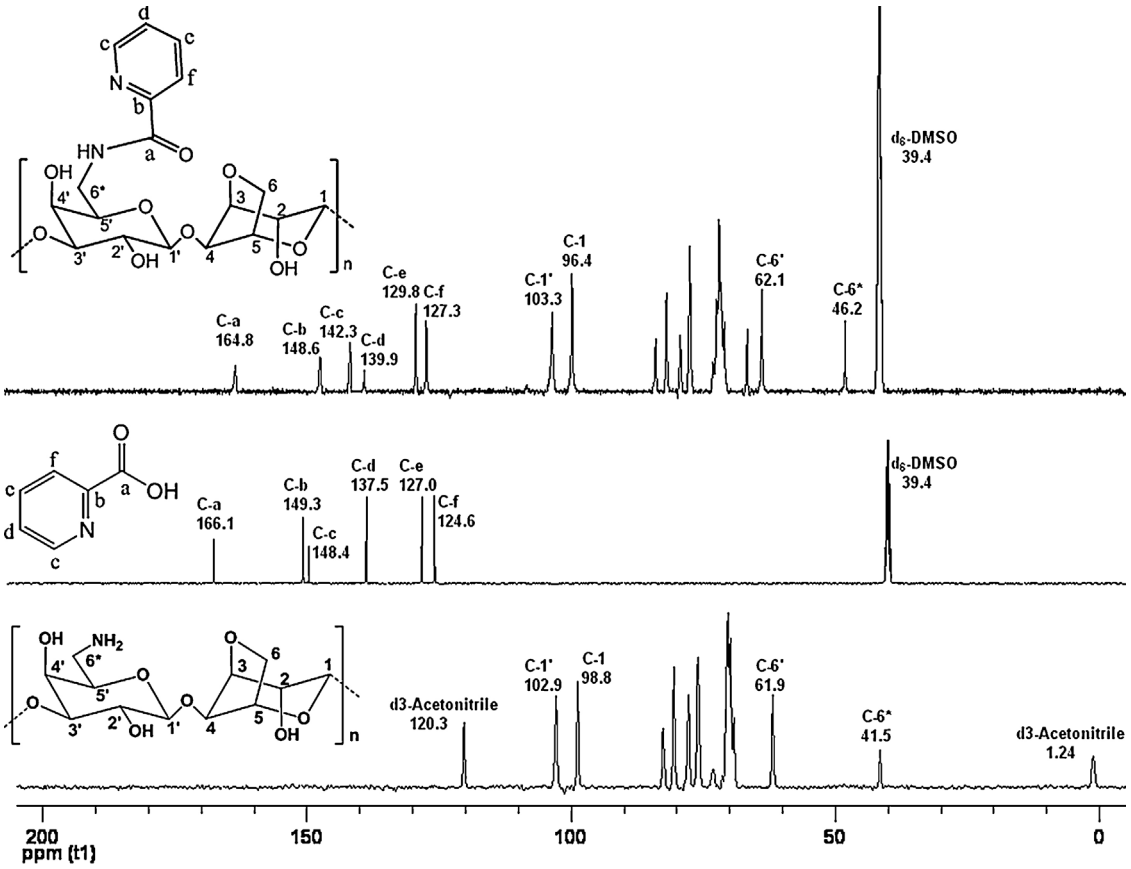


Fig. 3. ^{13}C NMR spectra of aminoagarose, picolinic acid, AA-PA in D_2O (60 mg mL^{-1}) at ambient condition on 125 MHz spectrometer, d_3-acetonitrile and d_6-DMSO was used as internal standard.

4.59–3.14 ppm (Fig. S9b). The aromatic protons of AA-NA appeared at 7.51–9.00 ppm, whereas these protons in nicotinic acid appeared at 7.52–9.06 ppm, similarly the aromatic protons of AA-PA appeared at 7.24–7.71 ppm, whereas these protons in picolinic acid appeared at 7.59–8.70 ppm, (Fig. S10b and c). This body of data confirmed the formation of AA-NA and AA-PA in the synthetic reaction.

3.3.4. UV–vis spectra

The non-modified agarose and AA did not exhibit any absorption maxima in the UV–vis region, while genipin exhibited absorption at λ_{max} (in distilled water, nm): λ_{max} 240 nm (ε 5000 L mol^{-1} cm^{-1}) The genipin cross-linked product G-AA showed absorption bands at λ_{max} (in distilled water, nm): 240 nm (ε 2680 L mol^{-1} cm^{-1}), 283 nm (ε 784 L mol^{-1} cm^{-1}), 606 nm (61.9 L mol^{-1} cm^{-1}). The appearance of absorption maxima at λ_{max} 240, 283 nm and 606 nm in G-AA indicated insertion of genipin creating cross-linked network engaging the free primary amino group of 6-amino agarose (Fig. S11). The new maxima at 606 nm had appeared due to the generation of an extended conjugation system as a result of genipin cross-linking manifesting the blue-color. This is in good agreement with those reported in the literature (Chhatbar et al., 2011). The cross-linking of genipin confirmed the replacement of hydroxyl group by an amino group on C-6 of agarose backbone. The nicotinic acid and AA-NA exhibited absorption at λ_{max} (in distilled water, nm): 262 and 260 nm (ε 5600 L mol^{-1} cm^{-1} & ε 1094.2 L mol^{-1} cm^{-1}). Similarly picolinic acid and AA-PA exhibited absorption at λ_{max} (in distilled water, nm): 262 and 265 nm (ε 6202.2 L mol^{-1} cm^{-1} and 2647.2 L mol^{-1} cm^{-1}), respectively. The appearance of absorption maxima at λ_{max} 260 nm in AA-NA and 265 nm in AA-PA indicated insertion nicotinic acid and picolinic acid on the aminoagarose backbone (Fig. S12) (Atac et al., 2012).

3.3.5. Optical rotation

The optical rotation values of parent aminoagarose $[\alpha]^{30\,^\circ C}_{589\,nm}$ (c 0.25, H_2O), nicotinic acid (c 0.25, H_2O), picolinic acid $[\alpha]^{30\,^\circ C}_{589\,nm}$ (c 0.25, H_2O) were −28.36°, +4.69, +0.475°, whereas those of aminoagarose-nicotinic acid $[\alpha]^{30\,^\circ C}_{589\,nm}$ (c 0.25, H_2O) and aminoagarose-picolinic acid $[\alpha]^{30\,^\circ C}_{589\,nm}$ (c 0.25, H_2O) were −31.50° and −10.40° respectively. The modified $[\alpha]^{30\,^\circ C}_{589\,nm}$ value of aminoagarose is significantly changes after chemically modification indicated changes in the molecular architecture as a result of functionalization with nicotinic acid and picolinic acid.

3.4. Scanning electron microscopy (SEM)

SEM images exhibited significant morphological differences between the AA, AA-NA, AA-PA products and the parent agarose (Fig. S13). Fig. 13a presents SEM image obtained for the parent aminoagarose, which appeared to be a cloud-like morphology. The morphologies of parent agarose, having cloud like morphologies got converted into an integrated and rod like structures in the AA-NA and AA-PA products as exhibited in their SEM images indicating formation of new compounds (Fig. S13).

3.5. Fluorescence measurements

The fluorescence emissions (λ_{max} 430 nm) of pure nicotinic acid, aminoagarose, AA-NA and (λ_{max} 412 nm) of pure picolinic acid, AA-PA were measured at 1×10^{-3} M concentration (Figs. 4 and 5). Parent agarose and aminoagarose at this concentration exhibited very low emissions. The emission spectrum of the modified aminoagarose recorded in distilled water (1×10^{-3} M) solution exhibited emission maxima ($\lambda_{em,max}$) at 430 nm by excitation

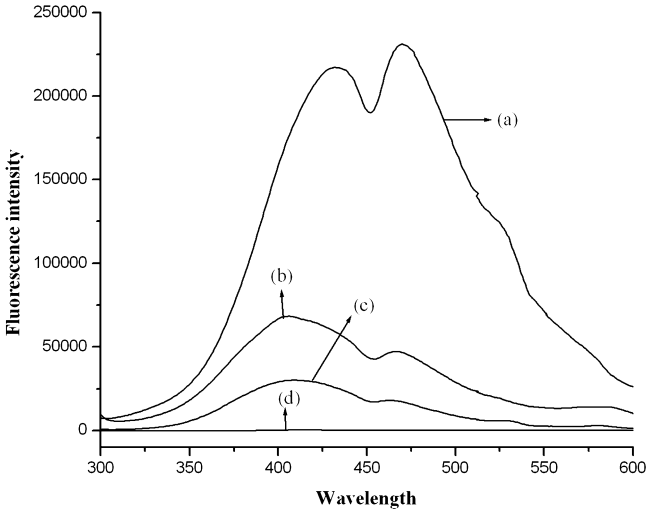


Fig. 4. Fluorescence emissions of (a) AA-NA, 1×10^{-3} M; (b) AA-NA, 1×10^{-4} M; (c) nicotinic acid, 0.2 mg in 1×10^{-3} M; (d) aminoagarose, 1×10^{-3} M.

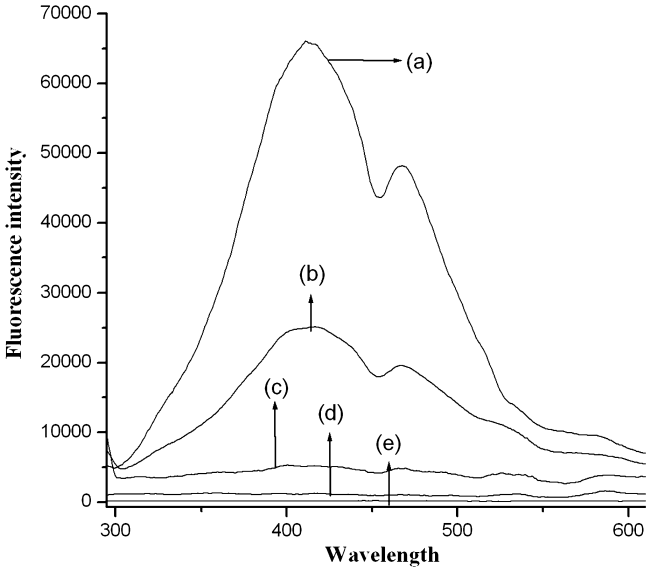


Fig. 5. Fluorescence emissions of (a) AA-PA, 1×10^{-3} M; (b) AA-PA, 1×10^{-4} M; (c) picolinic acid, 0.2 mg in 1×10^{-3} M; (d) aminoagarose, 1×10^{-3} M.

at 262 nm for AA-NA and ($\lambda_{em,max}$) at 412 nm by excitation at 262 nm for AA-PA. The fluorescence emissions (λ_{max} 430 nm) of the AA-NA (1×10^{-3} M) and emissions (λ_{max} 412 nm) of the AA-PA (1×10^{-3} M) in water were significantly higher (ca. 82%) and (ca. 90%) than those of the molar equivalents of NA and PA i.e. 0.2 mg present in their 1×10^{-3} M solutions respectively. This enhancement in fluorescence emissions may be attributed to certain molecular arrangements in the AA-NA and AA-PA derivatives facilitating intermolecular electron transfer processes including the π–π ones (Kondaveeti et al., 2013; Oza et al., 2012; Odo, Sogawa, Inoguchi, & Hirai, 2011).

4. Conclusions

A new 6-aminoagarose hydrogel has been synthesized in a facile microwave assisted Mitsunobu-inspired method. This hydrogel was found to be stable across a wide pH media e.g. 1.2, 7.0 and 12.5 for over 24 h. Like other prominent aminopolysaccharides e.g. chitin and chitosans (cf. Kim, 2011), the pH stability and high gel strength of 6-aminoagarose as compared to those of the parent agarose, it may be useful in various domains including glycotechnology and food applications as dietary fiber (Kadokawa et al., 2000; Furda, 1983, Chap. 8). The reactive amino group may be harnessed in synthesizing various materials targeting potential utilities involving the merits of amino and amide groups including affinity based separation technologies. Thus this 6-amino agarose may be treated as a precursor for synthesizing amino polysaccharide derivatives of interest. In fact this amino agarose was reacted with the bioactive pyridine carboxylic acids viz. pyridine-3- and pyridine-2-carboxylic acids to afford fluorescent new amide derivatives, which were characterized. This demonstrated the functional viability of the 6-amino agarose derivative.

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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.2014.02.051.

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