Polymer Journal, Vol. 37, No. 11, pp. 826–832 (2005)

Physical Modification of Agar: Formation of Agar-fatty Acid Complexes

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(Received June 2, 2005; Accepted July 27, 2005; Published November 15, 2005)

ABSTRACT: Four saturated (C12:0–C18:0) and one unsaturated fatty acid (C18:1) were allowed to react with agar and the resulting agar-fatty acid inclusion complexes were investigated. The bound fatty acids present in agar-fatty acid complexes were isolated and estimated by HPLC using UV–vis detector. Subsequently, confirmatory analysis of these fatty acids were done using TOF LC–MS technique. It was observed that the presence of fatty acids in the agar-fatty acid complexes affected the physical (*e.g.* gel strength, viscosity, swelling behaviour, syneresis and surface tension) and rheological behaviour (*e.g.* dynamic viscosity and viscoelasticity) of agar sol and gel.
[DOI 10.1295/polymj.37.826]

KEY WORDS Agar / Agar-fatty Acid Complex / Rheology / HPLC / TOF LC–MS /

Physical modification of starch to enhance various properties *viz.*, gelatinisation is well known. In many cases the starch is mechanically modified to provide improved properties.[1] Interaction of starch and surfactants are well studied. Lundqvist *et al.* have reported binding of surfactant to starch molecules by surface tension measurements.[2] Starch and fatty acid interactions in presence of whey protein is also reported by Zhang and coworkers.[3] The formation of three way complexes between starch and fatty acid has been widely reported and the presence of the fatty acid starch complex can be identified by size exclusion chromatography, large rapid visco analyser (RVA), thermo chemical measurements etc.[4] Extraction of free fatty acid from starch fatty acid complex by hot aqueous alcohol is reported, the use of less polar solvent like chloroform was found to be not suitable for such extraction.[5] Starch fatty acid interaction was mainly studied by isolating the complex from the plants and it was observed that starch form stronger complex with palmitic acid and weaker with linoleic acid.[6] Akuzawa and his coworkers have reported estimation of bound fatty acid by gas chromatography.[7]

Agar is a very important naturally occurring biopolymer. The backbone of this phycocolloids is made up of alternating 3-O-linked D-galactopyranose and 4-O-linked 3,6-anhydro-L-galactopyranose shown in Figure 1. The increased use of agar in various biological applications including biotechnology and molecular biology makes it more precious. It has been a constant endeavour of many research programmes to modify the physical and chemical properties of commercially important biopolymers. The extensive application of agar as gelling agent in many cosmetics and laxative formulations has made physical modification of agar all the more important. Preparation of low gel strength or creamy agar and its use as skin moisturiser is patented by Kojima and coworkers.[8]

This prompted us to undertake studies on the physical modification of agar by using fatty acids. Rheological, thermal and viscoelastic properties of the agar fatty acid adduct were studied. The most obvious effect was found in low gel strength and enhanced dynamic flow behaviour of the agar sol. The quantity of fatty acid in the agar fatty acid complex was determined by HPLC and LC–MS analysis of the methanol extract of the adduct. To our knowledge, the studies of interaction of agar-fatty acids have not so far been reported in the literature. This investigation provides a physical method of modification of agar. Such modified agars could be used in various cosmetic and health care product formulations, wherein both the gelling characteristics of agar as well as the merits of fatty acids could be beneficially exploited.

EXPERIMENTAL

Materials

Bacteriological agar was extracted from *Gelidiella acerosa* collected from Gulf of Mannar at Ervadi (9°15′N, 78°58′E), in March 2000 (Code No. AS0402909) adapting the method of Roleda *et al.*[9] The same agar sample was used for all experiments. The effects of various fatty acids on agar were studied. The following four saturated fatty acids and one unsaturated were used in the study: myristic (C14:0), palmitic (C16:0), stearic (C18:0), lauric (C12:0) and oleic acid (C18:1) from Sigma-Aldrich Inc. Wiscosin,

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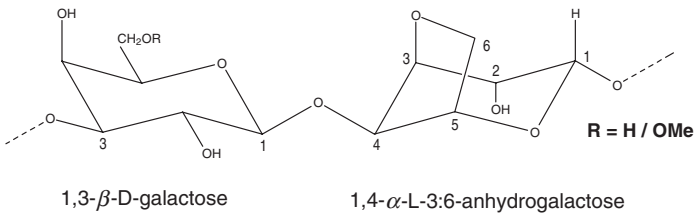


Figure 1. Repeating disaccharide units in agar.

USA. The fatty acids were used as received without further purification.

Measurements

The high performance liquid chromatography (HPLC) system used was a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan), including pumps 6AD, a Shimadzu SPD-10A UV–vis detector attached to a Shimadzu LC-10, chromatography manager. A Discovery C18 stainless steel column of 25 cm (length) × 4.6 mm (i.d.) packed with C18 reversed-phase material of 5 μm particle size, 300 Å pore size (SUPELCO, Sigma-Aldrich, Inc, USA) was used. The column was attached to a 5 cm C18 ODS guard column (Shimadzu, Kyoto, Japan). The samples were injected with a static injector (Rheodyne, Coati, CA, USA) and 20 μL microsyringe (Hamilton, Reno, Nevada, USA). Isocratic elution was performed at 50 °C with the solvent system, methanol: 5 mM phosphoric acid buffer, (9:1, v/v), at a constant flow rate 1.5 mL min^{-1}. The mobile phases were sonicated and filtered over 0.2 μm nylon filter (Cole-Parmer, USA). UV detection was carried out at 210 nm.

LC–MS Analysis

The liquid chromatography mass selective detector system used was Waters 2695 separation module with Waters 2487 dual λ absorbance UV–vis detector at 210 nm wavelength connected to a Waters Q-Tof micro YA-260 mass selective detector (MS/MS). Column used for LC was of Waters Symmetry C18 reverse phase column of 10 cm (length) × 4.6 mm (i.d.) with 3.5 μm particle size and 100 Å pore size. For the LC analysis methanol: water (9:1) was used as the mobile phase with 0.2 mL/min flow rate. The instrument parameters fixed were: Capillary voltage 2700 V, sample cone voltage: 30.0 V, extraction cone voltage: 0.5 V, Desolvation temperature 150 °C, source temperature: 80 °C, syringe rate 0.5 μL/min, ion energy 2.0 V, collision energy 7.0 V, using a Waters massLynx version 4.0 Mass analysis programme software.

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 solutions taking 1 mL sol on to peltier of the rheometer, for measurements in agar gel done at 25 °C. Viscosities at varying shear rate were studied at 45 °C. The temperature was maintained using the DC50 water circulator. Rheological data presented are means of three replicate measurements. Under the given experimental conditions no syneresis or slippage of gel was observed as there was no abrupt decrease in G′ values.[10]

Physical Properties

Measurement of pH was carried out using a model No. 535 pH meter from Systronics Scientific Instruments, India. Gelling and melting temperatures of agar gel in presence of various fatty acids were measured in the mixtures as described by Craigie *et al.*[11]

The gel strength (g cm^{-2}) was measured using a Nikkansui-type gel tester (Kiya Seisakusho Ltd. Tokyo, Japan). The measurements were performed on a 1.5% w/v agar solution at 20 °C (previously cured overnight at 10 °C) using a solid cylindrical plunger of 1 cm in diameter.[12] Apparent viscosity was measured at temperature (80 °C) using a Brookfield Viscometer (Synchrolectric Viscometer, Stoughton, MASS 02072). Spindle No. 1 and rpm 60 were used for measuring the apparent viscosity.

Thermal Measurements

Differential Scanning Calorimetric (DSC) and Thermogravimetric analysis (TGA) measurements were carried out on a Mettler–Toledo system with STAR^e software, from Switzerland. For this purpose, *ca.* 10 mg gels (1.5% w/v) were taken in an aluminum crucible weighing 40 mg and the measurements were done using temperature programme of 50 to 150 °C at 5 °C min^{-1} heating rate in an air atmosphere. TGA measurements were carried out using the temperature programme 40 to 110 °C at a heating rate of 2 °C per minute in an argon atmosphere, using sample mass of *ca.* 10 mg.

Surface Tension Measurements

Surface tension was measured by the static Wilhelmy plate using Dynamic Contact Angle Tensi-

Table I. HPLC analysis of the recovered fatty acid from the complex

Fatty acid and formulae	Retention Time (min)		HPLC Peak area for standard fatty acid	HPLC Peak area for the fatty acid recovered from the complex	Estimated quantity of fatty acid bound in the complex (in μg) per 100 mg agar
	Standard	Recovered			
Lauric acid ($C_{12}H_{24}O_2$)	3.436	3.072	1176236	180855	153.75
Myristic acid ($C_{14}H_{28}O_2$)	4.324	3.426	864958	263828	305.018
Palmitic acid ($C_{16}H_{32}O_2$)	9.211	10.433	2647633	378411	142.92
Stearic acid ($C_{18}H_{36}O_2$)	10.409	ND	1562236	Not detected	—
Oleic acid ($C_{18}H_{35}O_2$)	8.2	ND	ND	—	—

ometer (DCAT 21, Data Physics, Germany). To ensure removal of surface-active contaminants, all glass wares in contact with the sample were cleaned in chromic acid and rinsed with the double distilled water. The platinum plate was washed in double distilled water, heated in a Bunsen flame and left to cool at room temperature. The solutions are prepared with the double distilled water. Experiments are carried out in triplicate. For this measurement 0.05% w/v solution of the complex as well as control agar in double distilled water was prepared by heating the powder on a hot plate.

Swell Ability

Swelling properties of the agar and agar fatty acid complexes in water were measured by soaking the samples in water and measuring the weight gain in regular intervals as described by Park *et al.*[13]

Spectral Analyses

Infrared spectroscopy was recorded on a Perkin-Elmer Spectrum GX, FT-IR System, and USA by taking 2.0 mg of material in 600 mg of KBr (quantitative IR). All spectra were recorded taking average of two counts with 10 scans each with a resolution of 5 cm^{-1}.

For preparation of sols and gels of the complex for all the experiments, first the complexes were soaked in minimum quantity of hot methanol (*ca.* 50 °C) then desired amount of water was added. This method of preparation of samples was adapted for ensuring homogeneous dispersion of the adduct in water.

Syneresis Index

The amount of water exuded from the gel samples after standing for a certain period of time was determined and quantified using a modified method as described by Fiszman and Duran.[15] Approximately 10 grams of hot 1.5% (w/w) agar and agar fatty acid complexes were poured into test tubes (21 mm dia) and allowed to gel at room temperature (31–33 °C), and kept at 5 °C for 24 h. The initial weights of these gels were measured before placing them on dry Whatman (No. 1) filter papers. Weighing the gels after 2 h monitored the loss of water from the gels. The syneresis index values of the gel samples were taken as the difference between the initial weight of the gel and its final weight after 2 h. This value indicates the water holding capacity of the gel. The extent of syneresis was estimated by the amount of water Δw separated from gel phase. The degree of syneresis was estimated by $\Delta w/w_o$, where w_o stands for the initial weight of gel. All measurements were done in triplicates.

RESULTS AND DISCUSSION

HPLC Analysis

To a solution of fatty acid (100 mg) in methanol, agar (5 g) was added. The mixture was stirred at 50–60 °C temperatures for 5 h. The reaction mixture was filtered to separate the solid product, which was washed with excess hot methanol to remove adhering free fatty acid. The agar-fatty acid adduct (100 mg) was extracted (×4) by methanol on a Soxhlet apparatus for 4 h. The extractive was evaporated to dryness on a rotary evaporator. The residue, the amount of bound fatty acid component of the adduct, was subjected to HPLC analysis.[14] The amount of bound fatty acid to the agar was determined on the basis of the area under the peaks of standard and recovered fatty acids (Table I). Completion of the extraction of fatty acid was ensured by HPLC of the extract, wherein no peak of fatty acid was observed.

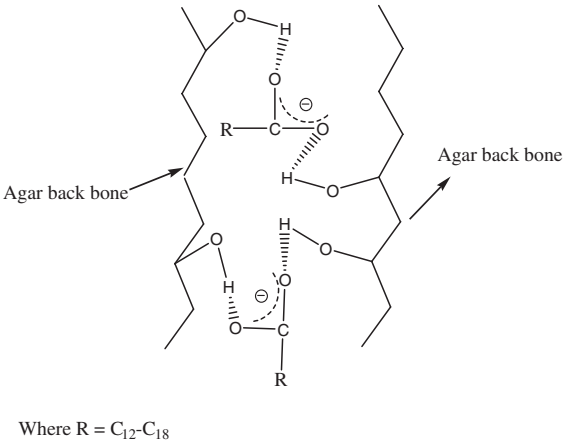
The agar that remained after extraction of fatty acids was dispersed in water (0.05%, w/v) by boiling and the surface tension of the dispersion was measured to be 59 mN/m at 27.1 °C, which is identical with the control agar dispersion at same concentration (Table II) confirming thereby the complete extraction of fatty acid from the adduct.

For HPLC analysis of standard fatty acids 100 mg

Table II. Surface tension study of the complexes

Sample (0.05% w/v)	Surface tension at 27.1 °C in water (mN/m)	Surface tension at 27.1 °C in 1:10 methanol/water (mN/m)
Double distilled water	64	27.5*
Control agar	59	26.2
Lauric acid + agar	36	21
Myristic acid + agar	39	19
Palmitic acid + agar	31	16
Stearic acid + agar	54	27
Oleic acid + agar	37	17

*Surface tension of 1:10 methanol water mixture.



Scheme 1. Schematic representation of the formation of fatty acid agar complex.

of the acid was dissolved in 2 mL of methanol and 20 μL of this solution was injected. For estimating free fatty acids in the complex, 100 mg of the fatty acid-agar complex was Soxhleted with methanol and the extract was evaporated to dryness. The residue was further dissolved in 2 mL methanol from which 20 μL aliquot was injected for HPLC analysis. It was observed from the HPLC analysis of the extracted free fatty acids from the agar-fatty acid complexes that tiny quantities (142.92 to 305.02 μg per 100 mg of agar-fatty acid complex) of fatty acids were bound to agar forming the complex. Stearic acid (C18) did not show any binding with agar, this may be due to the larger size of stearic acid, preventing this from getting accommodated in the molecular structure of agar (pleases see Scheme 1). On the contrary, myristic acid (C14) got bound in the maximum quantity (Table I), in the series of the fatty acids investigated, presumably because of its smaller size. Oleic acid was not detected in HPLC analysis where UV–vis detector was used. But the same was detected in the TOF LC–MS analysis (Figure 2), which may be due to higher sensitivity of the MSD system. The extractive obtained after the Soxhlet extraction of the adduct with methanol, was evaporated to dryness on a rotary evaporator. The residues were subjected for LC–MS analysis. Negative electron spray mode (ES^-) was used for the analyses. The base peaks (M-1) obtained for each of the extracted fatty acids tallied with the respective molecular weights of the fatty acids. These data reaffirm the phenomenon of binding of fatty acids to agar moiety.

It was observed that the presence of all the fatty acids, except stearic acid, decreases the gel strengths and viscosities (of the sol) of the complex by up to 120 g/cm^2 and 8 cP respectively. No effect of fatty acid on the gelling point of the complex was observed with respect to the control sample, whereas a lowering of melting temperature by up to 5 °C was observed (Table III).

Decrease in apparent viscosity and gel strength values for agar gel in presence of fatty acids, this effect is similar to that reported that in the case of starch.[7]

Surface Tension Measurements

Values of surface tension for agar (0.05% w/v) and agar-fatty acid complex in water and 1:10 methanol water mixture at 27.1 °C are depicted in Table II. Considerable decrease in the surface tension values for agar in presence of Lauric, myristic, palmitic acid indicates induced surface activity to agar molecule in presence of fatty acids. Stearic acid has no effect on the surface property of agar as evidenced from surface tension measurements.

Syneresis

The extent of syneresis as measured by the degree of syneresis values of the agar gel and the fatty acid agar complexes as shown in Table IV. It was observed that agar gel in presence of myristic acid showed maximum syneresis and least in presence of stearic acid. This is evident that agar gel in presence of myristic acid become more water repellent on standing *i.e.*, water holding capacity of agar gel decreases in presence of myristic acid. This attributes to the enhancement of hydrophobicity of the complexes.

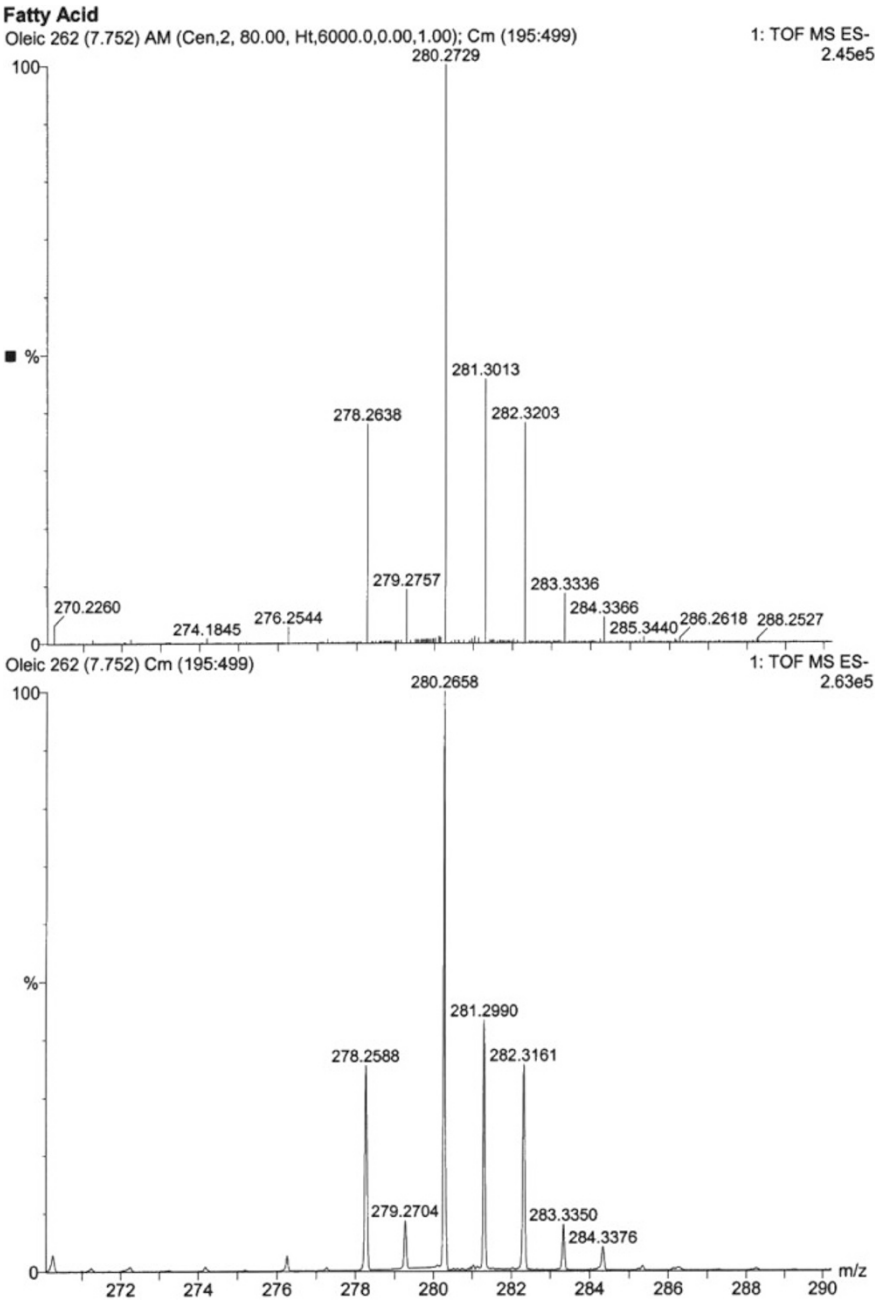


Figure 2. Mass fragmentation pattern of oleic acid isolated from the agar-oleic acid adduct.

Swelling Behaviour

Swelling behaviour of agar and agar fatty acid complexes in water is depicted in Figure 3. Dry samples (2 g each) were soaked in water and the weight gained by the samples were monitored and recorded at regular intervals. It was observed that swelling of agar-lauric acid adduct was maximum and it was lowest in the case of agar-myristic acid adduct. These results confirm that agar-fatty acid complexes are more hydrophobic than agar itself.

Dynamic Viscous Behaviour

Flow behaviour: Shear viscosity was measured under applied shear rate from 5–2301/s. It was observed that agar myristic acid complex has highest gel thinning property. This result indicates myristic acid imparts more fluidity into agar sol. On the other hand palmitic acid impart less fluidity and stearic acid did not induce any fluidity to agar sol. The property of decrement in dynamic viscosity of agar in presence of myristic and palmitic acid can be utilized to prepare low gel strength agar and there by may be used in cosmetic formulations (Figure 4).

Dynamic Viscoelasticity

Frequency dependence of G' and G'' for agar and agar fatty acid complexes in gel state are shown in Figure 5. Both moduli decrease for the complexes

Table III. Physical properties of the agar-fatty acid complex

Properties	Apparent Viscosity (Cp at 80 °C)	pH (80 °C)	Gel Strength (g/cm^2)[a]	Gelling T (°C)	Melting T (°C)
Control agar sol/gel (1.5% w/v)	35	6.9	860	40	88
Lauric acid + agar	27	6.5	760	39	85
Myristic acid + agar	29	6.7	740	39	83
Palmitic acid + agar	30	6.7	770	40	83
Stearic acid + agar	35	6.9	850	40	88
Oleic acid + agar	27	6.8	750	38	86

[a]Gel Strength was measured at 20 °C.

Table IV. Degree of Syneresis for agar gel and agar fatty acid complexes

Sample	Initial (w_o) weight (g)	Final weight (g)	Syneresis (Δw)	Degree of Syneresis ($\Delta w/w_o$)
Control agar	10.58	9.37	1.21	0.117
Agar + lauric acid	10.66	9.18	1.48	0.134
agar + myristic acid	10.64	9.11	1.53	0.143
agar + palmitic acid	10.55	9.20	1.53	0.127
agar + oleic acid	10.50	9.18	1.32	0.125
agar + stearic acid	10.54	9.35	1.19	0.112

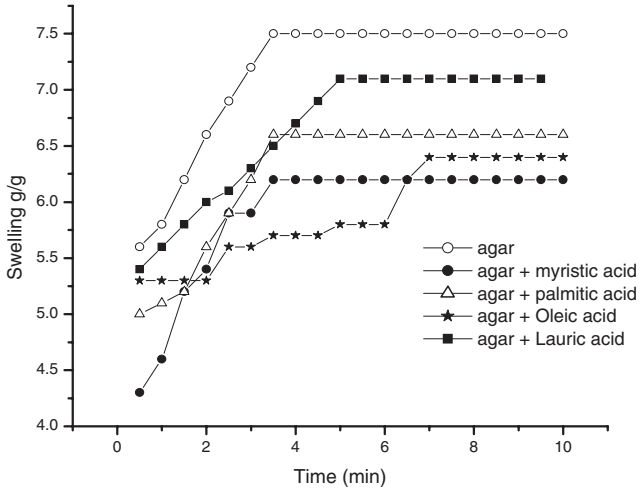


Figure 3. Swelling behaviour of agar and agar fatty acid complexes.

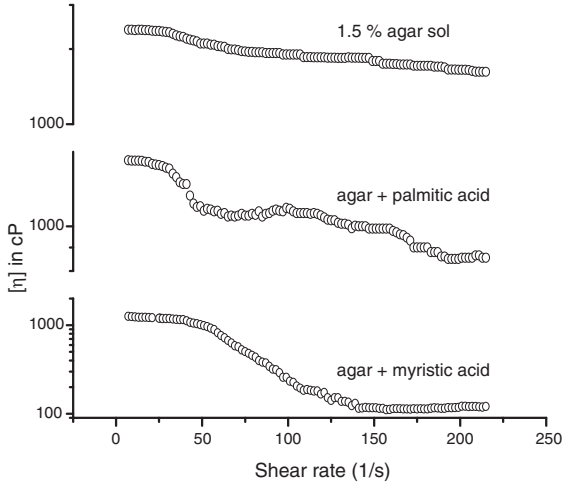


Figure 4. Dynamic viscosity profile for agar and agar fatty acid complexes in sol.

with myristic, lauric and palmitic acid and become more frequency dependent imparting more liquid like behaviour.

Spectral Analyses

No characteristic IR band for fatty acids was observed in the agar-fatty acid complex as well as in the physical mixture of agar and fatty acid (in the quantity that is present in the adduct). Therefore, the nature of binding of fatty acid and agar is not known. The fatty acid in the adduct can be extracted with refluxing methanol. Therefore, it appears that there exist weak

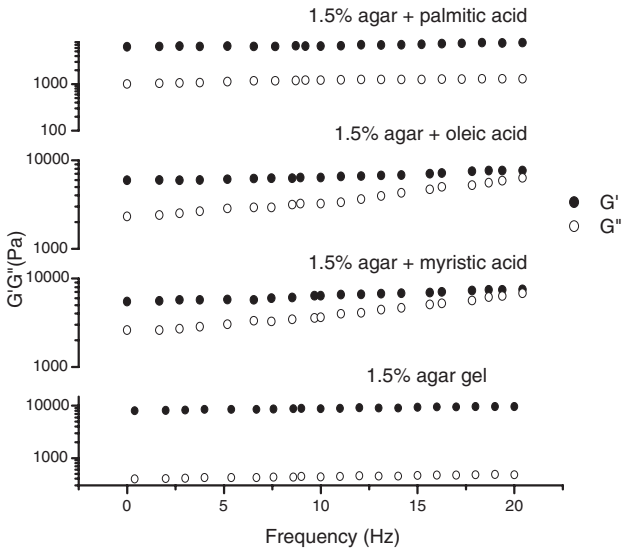


Figure 5. Frequency dependence of G′ and G″ for agar and agar fatty acid complexes.

interactive forces like van der Waals force, hydrogen bonding, rather than one of chemical nature. A schematic representation of the fatty acids incorporation in to agar structure is shown in Scheme 1.

Thermal Properties

Both TGA and DSC results did not show any changes in the respective thermograms both for agar and for the agar-fatty acid complex. It can, therefore, be concluded that inclusion of little amount of fatty acid inside the structure of the agar imparts no effect on its thermal properties in the solid state.

SUMMARY

It is concluded from the above studies that fatty acids bind to agar resulting in the modification of the physical and rheological properties of agar. Amount of bound fatty acid in the complexes were confirmed by TOF LC–MS and was estimated by quantative HPLC. It has been observed that fatty acids imparts more gel thinning on agar gel and this effect may be utilized to explore newer application of agar in cosmetics and heath care where merits of fatty acids can be beneficially exploited.

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