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Highlights

- Exploitation of waste algal biomass as source of ulvansulphated polysaccharide
- p(NIPAAm) graftedontoulvanacrylatemacroinitiatorby radical polymerization
- Thermosensitiveulvan-based hydrogels by using UV light by a straightforward method
- Thermogelling properties feasibleas in-situ hydrogel for biomedical applications

*Response to Reviewers

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Design, preparation and characterization of ulvan based thermosensitive hydrogels.

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Abstract

The present study is focused on the exploitation and conversion of sulphated polysaccharides obtained from waste algal biomass into high value added material for biomedical applications. ulvan, a sulphated polysaccharide extracted from green seaweeds belonging to *Ulva sp.* was selected as a suitable material due to its chemical versatility and widely ascertained bioactivity. To date the present work represents the first successful attempt of preparation of ulvan-based hydrogels displaying thermogelling behaviour. ulvan was provided with thermogelling properties by grafting poly(N-isopropylacrylamide) chains onto its backbone as thermosensitive component. To this aim ulvan was properly modified with acryloyl groups to act as macroinitiator in the radical polymerization of N-isopropylacrylamide, induced by UV irradiation through a "grafting from" method. The thermogelling properties of the copolymer were investigated by thermal and rheological analyses. Sol-gel transition of the copolymer was found to occur at 30-31°C thus indicating the feasibility of ulvan for being used as *in-situ* hydrogel forming systems for biomedical applications.

Keywords: ulvan, UV photopolymerization, poly(N-isopropylacrylamide), thermogelling, injectable systems.

1. Introduction

In recent years the scientific interest toward bio-based polymers is gaining a tremendous growth and it is estimated to increase steadily in the future (Babu, O'Connor & Seeram, 2013). Natural polymers represent a valuable platform of materials for replacing synthetic polymers of petroleum origin since their abundance and renewability are both constituting valid tools to limit the uncontrollable depletion of fossil resources. The rational exploitation of waste biomasses could represent the most promising strategy to create a sustainable system for the production of energy and materials. To that aim the application of modern biorefinery would allow for the conversion of waste materials, whose

environmental impact upon disposal is often a major concern, into a source of high value added materials. Natural materials from waste biomasses have been recently subjected to the attention of research community in many areas of interests. Waste lignin has been thoroughly investigated in the development of green hydrogels due to its inherent bioactivity (Thakur &Thakur, 2015) and high performance composite applications due to its reinforcing capability (Thakur, Thakur, Raghavan & Kessler, 2014). Cellulosic banana fibres obtainable as by-product of industrial waste have been explored as reinforcing components in polymer composite materials (Pappu, Patil, Jain, Mahindrakar, Haque & Thakur, 2015) as well as natural cellulose (Thakur & Thakur, 2014a; Thakur, Thakur & Gupta 2014). *Plantago Psyllium*, an annual plant distributed in most regions of the world, has been recently investigated as resource of bioactive polysaccharides at very low cost whose exploitation revealed promising in the preparation of hydrogels for miscellaneous applications (Thakur & Thakur, 2014b). Chitosan, a polysaccharide largely obtainable from seafood industry waste, represents so far the most studied biomaterial due to its outstanding chemical properties and biological activities. It is easily recovered from chitin, a nitrogen containing polysaccharide present in the exoskeleton of invertebrates, which is the second most ubiquitous natural polysaccharide after cellulose on earth. Due to the presence of amino groups chitosan represents a unique polysaccharide with outstanding potentiality for being chemically modified according to the desired application (Thakur & Thakur, 2014c). Among natural resources algal biomass is especially promising due to its abundance, rapid growth rate and wide availability of valuable materials (Jiao, Yu, Zhang, Ewart, 2011) and energy precursors (Mata, Martins & Caetano, 2014). Its extensive and rational exploitation would help to solve stringent environmental concerns such as uncontrollable growth and accumulation over seashores and landfills (Fletcher, 1996)

The green seaweed *Ulva*. could represent a valid platform of materials since it proliferates fast and occurs abundantly worldwide (Trivedi, Gupta, Reddy & Jha B, 2013). To date it is mainly used for food consumption and as nutritional supplement in East Asian countries such as China and Japan (Silva, Vieira, Almeida & Kijjoa, 2013). Recently the interest toward *Ulva* is gaining a tremendous increase due to the beneficial effects provided by the bioactive compounds constituting it (Silva, Vieira, Almeida & Kijjoa, 2013). Most of the biological activity displayed by *Ulva* has been found to be linked to its content of sulphated polysaccharide commonly labelled as ulvan (Chiellini & Morelli, 2011).

Ulvan could represent a promising platform of materials suitable for different applications comprising the biomedical field, but to date its feasibility as biomedical polymer is limited to some recent papers. Ulvan was especially investigated for the preparation of polymeric scaffolds for tissue engineering

applications by means of UV crosslinking (Morelli & Chiellini, 2010, Dash, Samal, Bartoli, Morelli, Smet, Dubruel & Chiellini, 2014), chemical crosslinking (Alves, Sousa, & Reis, 2013) and formation of hybrid materials as polyelectrolyte complexes (Barros, Alves, Nunes, Coimbra, Pires, & Reis, 2013, Toskas, Heinemann, Cherif, Hund, Roussis & Hanke, 2012, Alves, Duarte, Mano, Sousa, & Reis, 2012). Ulvan has been employed for the preparation of 2D polymeric membranes for drug delivery applications by means of chemical crosslinking (Alves, Pinho, Neves, Sousa, & Reis, 2012) and physical blending with poly(vinylalcohol) (Toskas, Hund, Laourine, Cherif, Smyrniotopoulos & Roussis, 2011). Moreover it was also investigated as coating material for medical grade PVC to provide its surface with antibacterial activity (Bigot, Louarn, Kebir & Burel, 2013). To the best of our knowledge the only method reported by the literature for the preparation of in situ gelling hydrogels based on ulvan is based on UV irradiation (Morelli & Chiellini, 2010).

In situ forming hydrogels represent an excellent tool to overcome drawbacks typically encountered by using preformed scaffolds (Ruel-Gariepy & Leroux , 2004) since they can be introduced in a minimally invasive manner in the body and fill perfectly the defects present in the site of action. Such hydrogels are of particular interest because drugs, proteins, and cells can be easily incorporated into polymer solutions prior to administration. The difference between the body and room temperature represents a natural stimulus that is commonly used in the preparation of *in situ* gelling systems.

In the present paper is reported the first attempt to develop thermosensitive hydrogels based on ulvan. Thermogelling is an advantageous strategy for the preparation of hydrogels whose applications require shorter times of degradation and weaker mechanical properties since the constituting networks are based on weak physical crosslinks.

In order to provide ulvan with thermogelling behaviour poly(N-isopropylacrylamide) (pNIPAAm) was grafted onto the polysaccharide backbone as pendant chains. p(NIPAAm) is a well known temperature sensitive polymer whose aqueous solutions exhibit phase transitions from solution to gel at lower critical solution temperature (LCST) of about 32°C due to a structural rearrangement of the polymeric chains (Klouda & Mikos, 2008; Ha, Lee, Chong & Lee, 2006).

p(NIPAAm) was grafted onto ulvan by radical polymerization of N-isopropylacrylamide onto pendant acryloyl groups properly conjugated to the polysaccharide to act as chain initiator. Acrylate ester groups were selected as double bond precursors due to their sensitivity to radical polymerization and susceptibility to hydrolytic degradation. The radical polymerization was induced by UV irradiation in order to prepare the copolymer by straightforward method easily reproducible on a large scale.

The prepared copolymer was fully characterized by chromatographic, spectral and thermal analyses. The thermogelling behaviour was objectively assessed by thermal and rheological analysis and by macroscopically observing the occurring of the phase transition from liquid to solid of the relevant solutions at different temperatures.

2. Experimental

2.1 Materials

Ulvan batch in powder as extracted from *Ulva armoricana* was kindly supplied by CEVA within the framework of the EU-founded project BIOPAL. N-Isopropylacrylamide (NIPAAm) (Sigma-Aldrich) was purified by recrystallization from hexane and dried under vacuum. Acryloyl chloride (97.0%) was purchased from Sigma Aldrich and used as received. Phosphate Buffer Saline (PBS) 10mM, pH 7.4 was prepared by dissolving 0.2 g of KCl, 0.2 g of KH_2PO_4, 8.0 g of NaCl and 3.8 g of $Na_2HPO_4 \cdot 12H_2O$ in 1 liter of deionized water. The final pH was adjusted to 7.4 with NaOH 5 N and the resulting solution was steam sterilized (121°C for 20 min) before use and storage. All reagents and solvents used were of analytical grade and obtained by Sigma-Aldrich.

2.2 Synthesis

2.2.1 Preparation of ulvan macromer

Ulvan-Acrylate conjugate (UA) was prepared through esterification reaction between the hydroxyl groups of ulvan and acryloyl chloride (AC) by following a procedure reported by the literature for the synthesis of acryloyloxystarch [Jantas, 1997].

In a 250 ml round bottomed, three-necked flask provided with a magnetic stirrer 2.0 g of ulvan (5 mmol of disaccharide repeating units corresponding to 15 mmol of reactive hydroxyl groups) were dissolved in 40 ml of deionized water. The resulting solution was stirred overnight to allow for the complete polysaccharide dissolution. An aqueous solution containing NaOH (17.6 g; 440 mmol) and 2-butanone (8 ml) were subsequently added at 4°C and the obtained solution was kept stirring for 30 minutes to promote the activation of the hydroxyl groups of ulvan. A solution of acryloyl chloride (32 ml; 395 mmol; 26:1 molar ratio of AC to reactive ulvan hydroxyl groups) in toluene (40 ml) was added dropwise at 4 °C to the reaction mixture and the obtained solution was kept under magnetic stirring for 3 h in the same conditions.

The aqueous phase containing the modified ulvan was purified by twice precipitation into absolute ethanol (1:10 v/v). After centrifugation the product was repeatedly washed with absolute ethanol and diethyl ether and then dried under vacuum. The obtained product was further purified by exhaustive dialysis against deionized water (Cellulose Ester, MWCO = 10000, Spectra/Por® Biotech) and freeze-dried at -50 °C. Typical product yields ranged from 60 to 65 %.

The spectroscopic characterizations of the obtained solid confirmed the successful preparation of the desired product.

2.2.2 Preparation of poly (N-isopropylacrylamide)-g-ulvan (UA-NIPAAm)

A solution containing 0.25 mg of 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (IRGACURE® 2959), 25.0 mg of ulvan-Acrylate macromer and 50.0 mg of purified N-isopropylacrylamide in 5 ml of deionized water were prepared in 25 ml glass containers and exposed to UV source (400 W high pressure mercury arc, 365 nm, 8-10 $mW \cdot cm^{-2}$, Helios Italquartz) for 30 minutes.

The copolymers were then purified by exhaustive dialysis against water for 3 days and freeze dried at -50 °C. Typical product yields ranged from 70 to 75%.

2.2.3 Preparation of poly (N-isopropylacrylamide) (pNIPAAm)

A solution containing 0.25 mg of 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (IRGACURE® 2959) and 50.0 mg of purified N-isopropylacrylamide in 5 ml of deionized water were prepared in 25 ml glass containers and exposed to UV source (400 W high pressure mercury arc, 365 nm, 8-10 $mW \cdot cm^{-2}$, Helios Italquartz) for 30 minutes.

The copolymers were then purified by exhaustive dialysis against water for 3 days (Cellulose Ester, MWCO = 10000, Spectra/Por® Biotech) and freeze dried at -50 °C. Typical product yields ranged from 60 to 65%.

2.3 Characterization

2.3.1 FT-IR analysis

FT-IR spectra were recorded on liquid films and KBr pellets (1/50 mg/mg) in the range of 4000–400 cm-1 by using a Jasco FT-IR 410 spectrophotometer with a resolution of 4 cm^{-1}. Each spectrum was recorded after 16 scans.

2.3.2 ^{1}HNMR analysis

NMR spectra were recorded on a Varian Gemini 200 spectrometer using a Sparc4 (Sun) console and VNMR6.1B software. Spectra were processed by using SpinWorks software (version 3.1.7.0). NMR spectra were recorded on 1–2% (w/v) solutions at 20 °C.

2.3.3 GPC analysis

GPC analysis was carried out with a Waters 600 model equipped with a Waters 410 Differential Refractometer and two Ultrahydrogel™ linear 6-13 μm columns (7.8 x 300 mm) (Waters, Milford, USA). operating at a column temperature of 30°C. Mobile phase constituted by 0.1 M sodium nitrate in water was eluted at a flow rate of 1 ml.min^{-1}.

Pullulan standards (Polymer Laboratories, UK) were used to obtain a calibration curve (range 6000-400000 g/mol).

2.3.4 DSC analysis

DSC analysis was performed by using a Mettler DSC-822 (Mettler Toledo, Milan, Italy) under an 80 ml/min nitrogen flow using samples of 5–10 mg. The samples were dried before analysis in vacuum at 70°C, and then kept in a desiccator. Each sample was initially heated to 185 °C, held isothermally for 1 min, cooled to 20 °C, and reheated to 185 °C. In all experiments the heating rate was set to 10 °C /min and the cooling rate to 20 °C/ min. The glass transition temperatures (Tg) were taken as the inflection point in the second heating cycle thermograms.

2.3.5 Determination of the thermogelling behaviour of (UA-NIPAAm)

2.3.5.1 Tilting Method

The sol-gel transition temperature for the UA-NIPAAm hydrogels was determined by using a vial *tilting method*. The gel state was determined by inverting the vial when no fluidity was visually observed in 1 minute [Jin et al., 2009].

Samples containing UA-NIPAAm at different concentrations (2-5 wt%) were tested in PBS (10 mM, pH 7.4) at the following temperatures: 25°C, 34°C, 37°C.

2.3.5.2 Determination of the Lower Critical Solution Temperature (LCST)

A set of solutions having different concentrations of UA-NIPAAm were prepared in PBS (10 mM, pH = 7.4) and analyzed by DSC to determine the LCST of the copolymer and to assess the influence of the copolymer concentration on the obtained LCST values.

Each solution was introduced into a standard aluminium crucible, and the crucible was hermetically sealed to avoid the evaporation of water. The DSC measurement was performed at a heating rate of 10°C/min in the temperature range from 10 to 60 °C. The temperature at the minimum of the endothermic peak was considered as the LCST value.

The same analysis was carried out on 4 wt% solution of UA-NIPAAm in deionized water to assess the influence of the ionic content of the dissolving medium on the LCST value of the copolymer.

2.3.6 Rheological analysis

Rheological measurements were carried out on PBS solutions (10 mM, pH 7.4) containing 4 wt% UA-NIPAAm by using a Rheometric Scientific Instruments, RM500 Rheometer (Lands, Sweden) equipped with a temperature-controlled steel bottom plate and using a plate to plate geometry (steel, 20 mm diameter). Data were processed by using the Rheoexplorer 3.0 software.

The rheological properties of the solutions were studied in the temperature range of 15 to 40 °C at a shear stress of 2 Pa, using a plate to plate gap of 0.052 μm and an angular frequency of 3.14 rad/s [Lian et al., 2012]. Measurements were performed by maintaining the samples for 10 minutes at a selected temperature (15°C, 20°C, 25°C, 30°C, 35°C, 37°C, 40°C) and collecting the data by regular intervals of 20 seconds each. The mean values of shear viscosity (η) and complex shear modulus (G*) were calculated for each analyzed temperature and reported as mean ± standard deviation based on the experiments run on 3 samples.

3. Results and discussions

Ulvan is a polysaccharide whose composition is heterogeneous and varies according to the taxonomic origin and the harvesting season of the algal biomass (Lahaye & Robic, 2007). However systematic studies carried out on the chemical composition of ulvan revealed that it is mainly composed by disaccharide repeating units constituted by uronic acid such as D-glucuronic or L-iduronic, linked to L-rhamnose-3-sulfate (Fig. 1) (Lahaye & Robic, 2007).

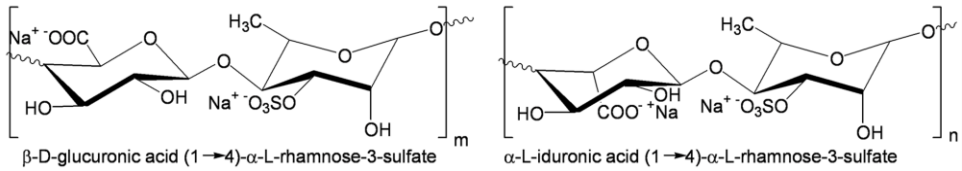

Fig. 1. Chemical structure of the main repeating units of ulvan

The availability of both hydrophilic (hydroxyl, carboxyl, sulphate) and hydrophobic (methyl) groups provides ulvan with unique properties that are not easily found in other polymers of natural origin (Robic, Gaillard, Sassi, Lerat & Lahaye, 2009). The chemical versatility of this material allows for a wide range of chemical reactions to obtain proper functionalization according to the desired application.

The strategy adopted for the preparation of thermosensitive poly (N-isopropylacrylamide)-g-ulvan (UA-NIPAAm) involved a two step processes comprising the modification of ulvan with acryloyl groups followed by the UV induced free radical polymerization of N-isopropyl acrylamide (NIPAAm) onto the acrylate moieties of the polysaccharide acting as chain initiators (Fig. 2).

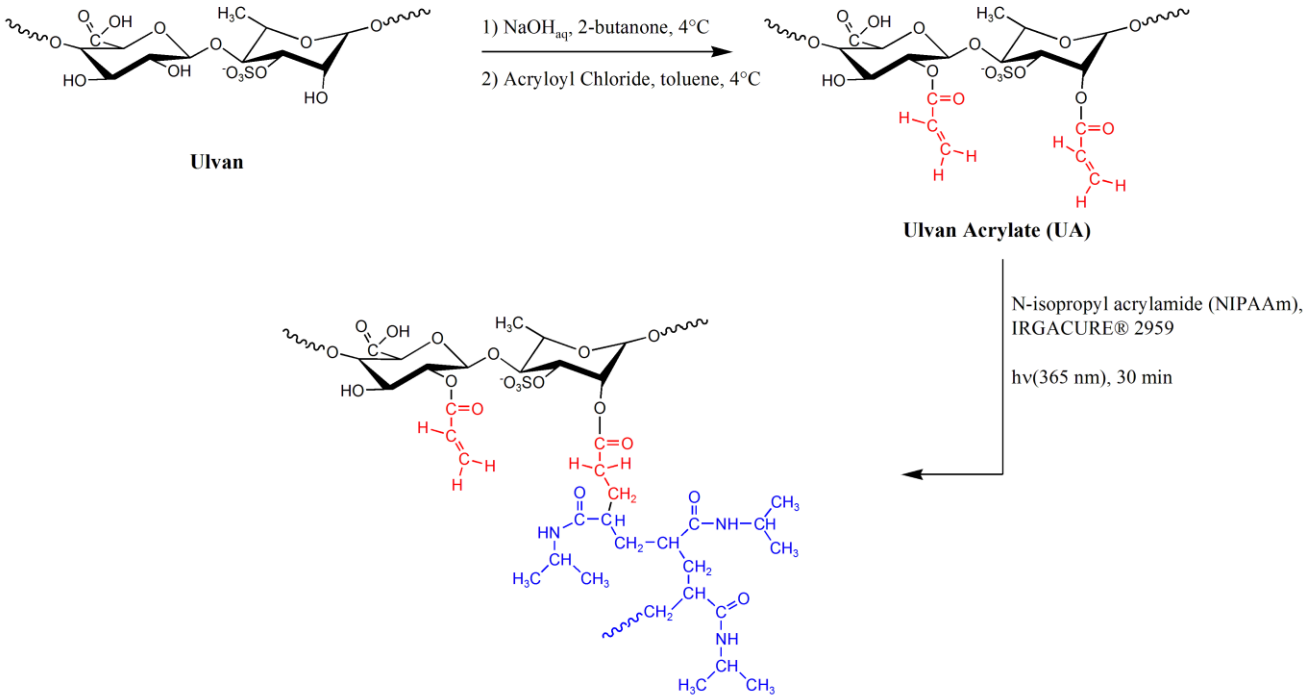

Fig. 2. Preparation of UA-NIPAAm

3.1 Preparation of poly (N-isopropylacrylamide)-g-ulvan (UA-NIPAAm)

3.1.1 Synthesis and characterization of ulvan-acrylate (UA)

The introduction of double bond groups into ulvan structure represented a strict requirement to be matched to promote the copolymerization of NIPAAm by means of radical processes. In a previous study we reported the functionalization of ulvan with alkenyl groups (Morelli & Chiellini, 2010). Ulvan was conjugated with methacryloyl moieties for the development of hydrogels covalently crosslinked by UV irradiation. Although the obtained hydrogels resulted coherent and stable the authors evidenced a limited reactivity of the methacryloyl groups during UV exposure postulating the antioxidant activity of ulvan as the possible cause (Hu, Geng, Zhang & Jiang, 2001). In order to boost the reactivity of ulvan toward UV mediated radical polymerization our strategy was to conjugate the polysaccharide with acryloyl groups. Indeed acrylates are considerable more reactive than methacrylates in radical processes due to steric and electronic effects.

Ulvan-Acrylate (UA) was prepared by esterification reaction of the hydroxyl groups of the polysaccharide by using acryloyl chloride as precursor according to a modified procedure reported by the literature for the preparation of acryloxystarch (Jantas, 1997). The reaction was carried out in a two phase solvent system by following the Schotten-Baumann reaction conditions. 2-butanone (MEK) was added to enhance the compatibility between the two immiscible phases. The reaction solution was left under stirring at 4°C in order to minimize the basic hydrolysis of the formed ester linkages and to avoid the polymerization of the acryloyl groups present in solution.

The excess of NaOH used was needed both to increase the nucleophilicity of the hydroxyl groups of Ulvan and neutralize the acid generated during the esterification reaction and the hydrolysis of the unreacted acryloyl chloride. Acryloyl chloride was used in large excess due to the low availability of the reactive hydroxyl groups of ulvan especially in basic conditions where the polymeric chains of the polysaccharides are usually dispersed in the form of beads and aggregates (Robic, Gaillard, Sassi, Lerat & Lahaye, 2009).

The successful incorporation of acryloyl groups onto ulvan structure was confirmed by FT-IR and ^{1}H-NMR spectroscopy. The FT-IR spectrum of ulvan acrylate (UA) revealed the presence of a peak at 1726 cm^{-1} not detected in the FT-IR spectrum of the pristine ulvan, ascribed to the carbonyl stretching vibration of an α,β-unsaturated ester bond (Fig. 3).

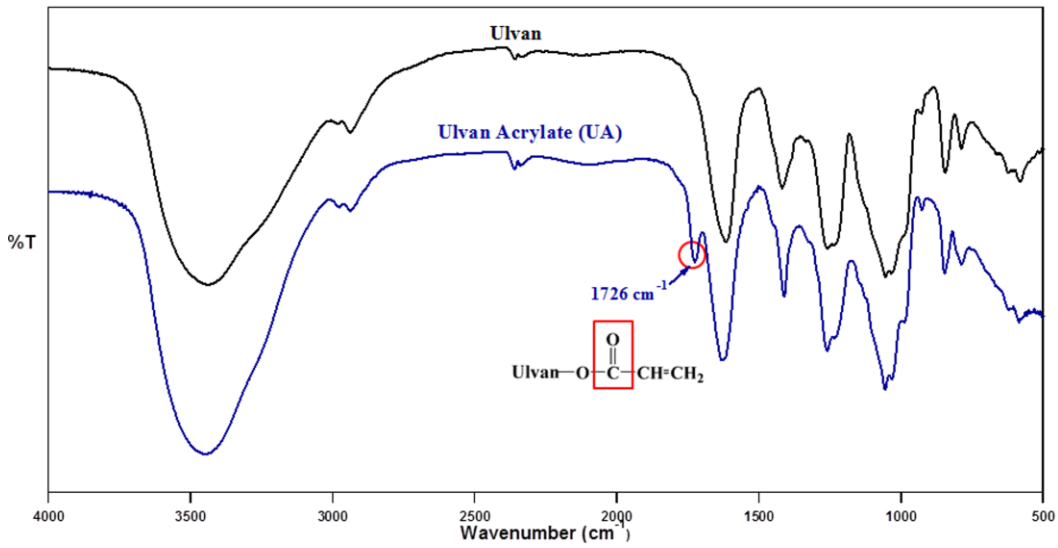

Fig. 3. Comparison of the FT-IR spectra of ulvan and ulvan acrylate macromer.

The ^{1}HNMR spectrum of UA recorded in D_2O unequivocally evidenced the presence of the peaks of alkenyl protons of acrylate (**b, c, d**, Fig. 4b) not found in the spectrum of the pristine ulvan.

The degree of substitution (DS) defined as the number of acryloyl groups per repeating units was determined by ^{1}H-NMR, by calculating the ratio of the area of the peaks relevant to the alkenyl protons of acrylate (**b**+**c**+**d**, Fig. 4b) to that of the methyl protons of ulvan (**a**, Fig. 4a). Typical obtained DS values ranged from 0.5 to 0.6 corresponding to one acryloyl group for every two repeating units of polysaccharide.

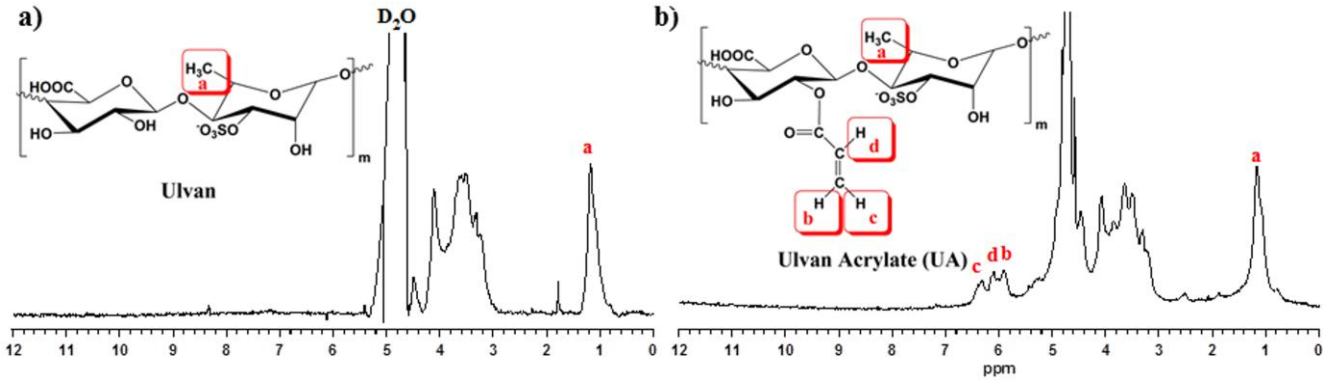

Fig. 4. ^{1}HNMR spectra with relevant peak attribution for DS calculation of a) ulvan and b) ulvan acrylate recorded in D_2O.

3.1.2 Synthesis and characterization of poly (N-isopropylacrylamide)-g-ulvan (UA-NIPAAm)

To the best of our knowledge, the preparation of thermosensitive hydrogels based on ulvan has never been reported by the literature. Our strategy was to provide the polysaccharide with thermogelling behaviour by using an easy and straightforward procedure starting from readily available precursors. The introduction of poly(N-isopropylacrylamide) chains as thermosensitive components into ulvan backbone by means of UV irradiation would undoubtedly favour the development of a convenient process due to the wide commercial availability of the (N-isopropylacrylamide) monomer and straightforward use of irradiation processes.

Two different approaches were potentially available to obtain the desired copolymer: 1) The 'Grafting to' approach based on the covalent conjugation of the two polymeric components, each one properly functionalized; 2) The 'Grafting from' approach requiring the polymerization of the desired monomer onto the other polymer acting as initiator (Zdyrko & Luzinov, 2011). The first approach was not taken into account since the covalent conjugation between two polymers could suffer from poor reactivity due to steric hindrance. Our strategy was to copolymerize NIPAAm monomer onto UA backbone by using the acrylate groups as radical chain initiators. The process was carried out in environmentally benign conditions by using water as solvent and IRGACURE® 2959 as a cytocompatible photoinitiator (Bryant, Nuttelman & Anseth, 2000). A large molar excess of NIPAAm was used in order to minimize possible covalent crosslinking of UA during the polymerization. The weight ratio of N-isopropylacrylamide/UA used for the preparation of the grafted copolymer was selected on the basis of a work regarding the preparation of thermosensitive chondroitin sulphate, a polysaccharide of animal origin that shares structural resemblance with ulvan (Varghese et al. 2008). Different times of UV exposure were investigated to optimize the experimental conditions and the yields of polymerization. Extensive times of UV irradiation led to the formation of a reversible gel within the polymerization vessel due to the thermogelling of p(NIPAAm) chains at the temperature aroused within the UV chamber and/or irreversible gel due to the covalent crosslinking between UA and p(NIPAAm). 30 minutes of UV irradiation proved to be the best compromise between gel formation and product yield. The copolymer was purified by extensive dialysis against water in order to remove p(NIPAAm) oligomers and unreacted NIPAAm monomer.

The FT-IR analysis of the polymer showed several distinctive peaks of both ulvan and poly(NIPAAm), indicating their presence in the purified materials (Fig. 5).

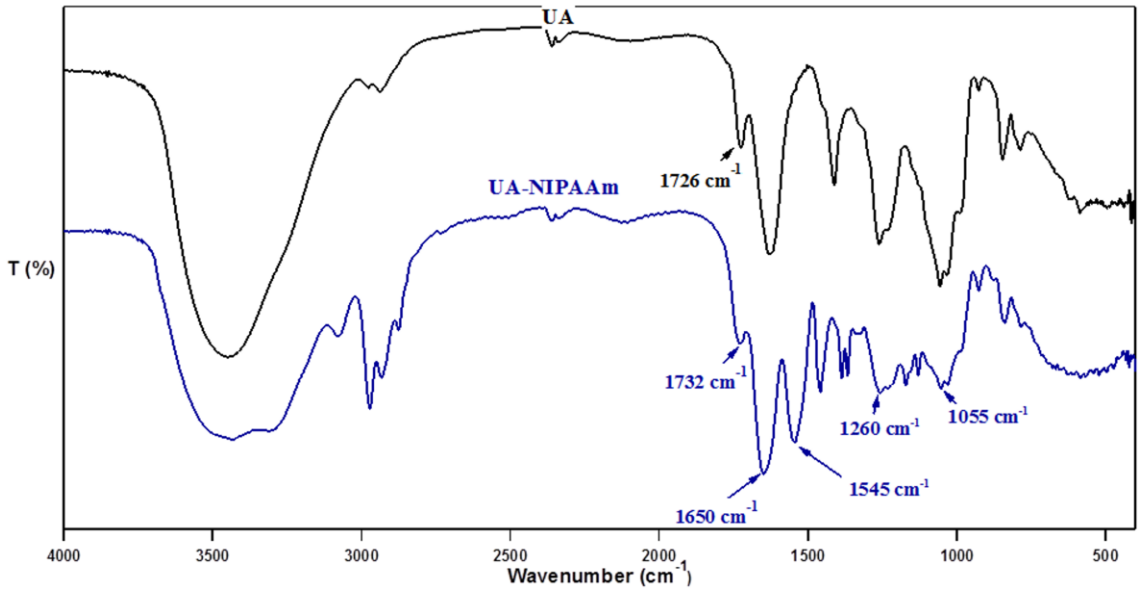

Fig.5. Comparison of the FT-IR spectra of ulvan acrylate (UA) and poly (N-isopropylacrylamide)-g-ulvan (UA-NIPAAm). The absorption wavenumbers evidenced within the picture represented most indicative peaks relevant to the presence of each polymeric component within the purified material.

The presence of ulvan was confirmed by the peaks at 1055 cm^{-1} and 1260 cm^{-1}due to the C-O-C stretching vibrations and the stretching vibration of S=O of sulphate of the polysaccharide respectively (Pengzhan , Ning, Xiguang , Gefei, Quanbin & Pengcheng, 2003).

The shift of the peak of the α,β-unsaturated ester bond of UA from 1726 cm^{-1} to 1732 cm^{-1} could be ascribed to formation of saturated ester bonds thus confirming the reactivity of the acrylate groups in the radical polymerization with NIPAAm (Scheme 1).

Characteristic absorptions of poly(NIPAAm) were found at 1650 cm^{-1} and 1545 cm^{-1} corresponding to the C=O stretching vibration (amide I band) and to the N-H bending (amide II band) of the polyamide.

The analysis ^{1}HNMR of the polymer was carried out at 20°C by using deuterated water as solvent. The spectrum mainly revealed the presence of p(NIPAAm) in the copolymer due to the overlapping of peaks with those characteristic of ulvan (Fig. 6). The peaks at 1.1 ppm and 3.8 ppm were ascribed respectively to the methyl and methine protons of the isopropyl group of p(NIPAAm). The peaks at 1.5 ppm and 1.9 ppm were assigned to the methylene and methine protons of the polymer backbone of p(NIPAAm) indicating the successful polymerization of the monomer**.** The presence of ulvan was evidenced by the broad absorption found in the range 3-4 ppm mostly superimposed by the peak **h** of p(NIPAAm) (Fig. 6). The absence of the peaks relevant to the acrylate groups (**b**, **c**, **d**, Fig. 6) indicated the occurring of their complete reaction during UV exposure. Under the adopted conditions they could

react either as initiator for the grafting polymerization of N-isopropylacrylamide to provide UA-NIPAAm or as a crosslinker by covalently reacting with further Ulvan acrylate groups. The absence of unsoluble material following UV irradiation indicated the exclusive formation of the grafted copolymer. By taking into account the complete reactivity of the acrylate groups of UA in the copolymerization with N-isopropylacrylamide, it can be reasonably assumed that the degree of grafting of p(NIPAAm) chains onto ulvan was nearly even to the degree of substitution of acrylate groups in the polysaccharide (DS = 0.5-0.6).

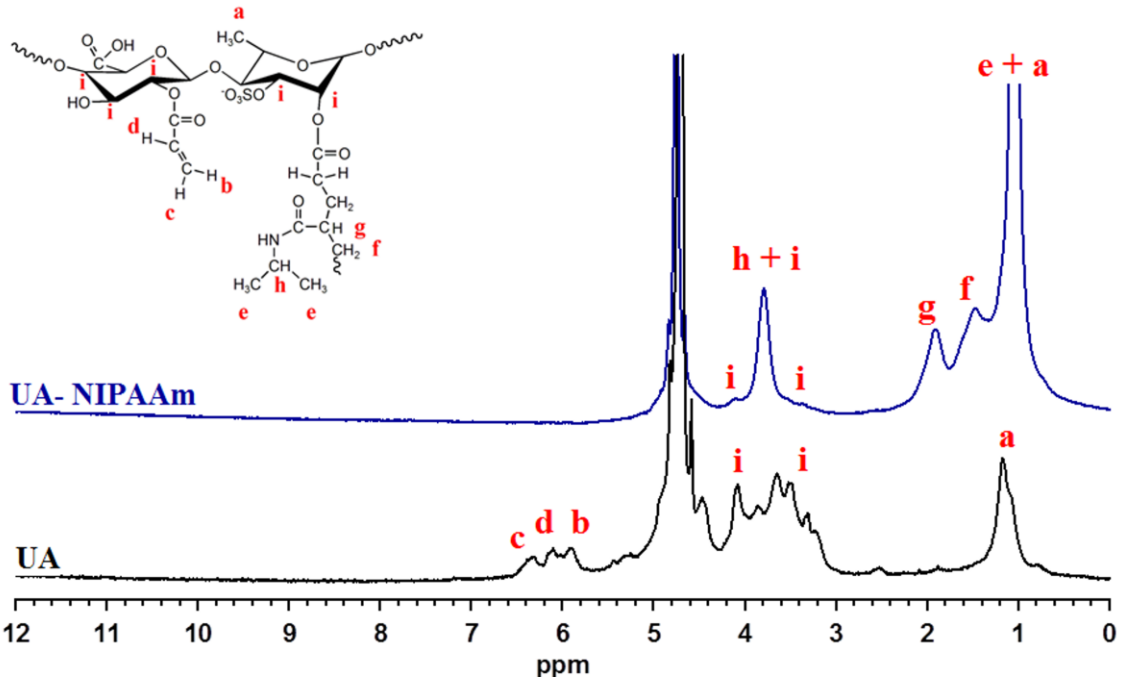

Fig. 6. ^{1}HNMR of UA-NIPAAm and UA with relevant peak attribution.

3.1.3 Thermal analysis

To rule out the hypothesis of physical mixture and further evidence the copolymeric nature of UA-NIPAAm the product was characterized by DSC analysis. The thermal behaviour of UA-NIPAAm was compared to that of the constituting homopolymers recorded both separately and as physical mixture. To gain objective data the homopolymers were prepared under the same experimental conditions as UA-NIPAAm and the composition of the physical mixture was set according to the same feed ratio of homopolymers used for the preparation of UA-NIPAAm (pNIPAAm/UA 2/1 w/w).

The DSC traces of the analyzed samples did not display the presence of melting peaks indicating that all the materials were completely amorphous (Fig. 7). The analyses were carried out on the second

heating cycle thermograms in order to erase the thermal history of the samples. The glass transition temperatures (Tg) taken at the inflection point of the curves, provided us with crucial information about the chemical nature of UA-NIPAAm.

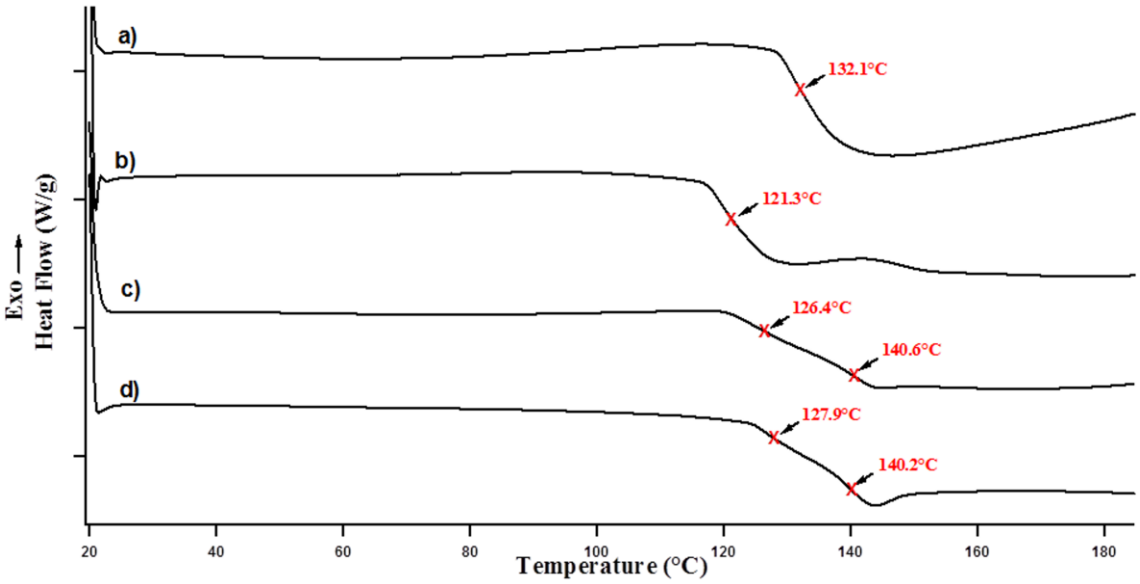

Fig. 7. DSC analysis of a) 30 minutes UV irradiated ulvan acrylate (UVUA), b) UA-NIPAAm, c) UVUA/pNIPAAm physical mixture, d) pNIPAAm. Tg values were taken at the inflection point of the second heating cycle curve.

Trace a) (Fig. 7) revealed the thermal behaviour of UA after being UV irradiated for 30 minutes at the same conditions employed for the other samples and it was taken as blank. The recorded transition was interpreted as being second order and the inflection point measured at 132.1°C as the glass transition of the irradiated material.

The thermogram of pNIPAAm (trace d), Fig.7) displayed the occurrence of two glass transitions temperatures likely due to the presence of crosslinked material within the sample. The DSC trace of the physical mixture (trace c) Fig.6) was very similar to that of p(NIPAAm) indicating a poor compatibility between the constituting homopolymers.

The thermogram of UA-NIPAAm (trace b) Fig.7) revealed the presence of a single glass transition temperature Tg whose value (121.3 °C) was lower with respect to those of the relevant homopolymers. The absence of a second Tg evidenced the enhanced compatibility of the two constituting homopolymers in UA-NIPAAm thus strongly supporting the hypothesis of the copolymeric nature of UA-NIPAAm. The lower Tg value recorded for UA-NIPAAm as compared to those of the relevant

homopolymers could be ascribed to a plasticizing effect induced by the grafted pNIPAAm chains (Jyothi, Carvalho, 2013).

3.1.4 Molar mass analysis

The recorded chromatograms revealed that all the analyzed materials were characterized by a broad molecular weight distribution (Fig. 8).

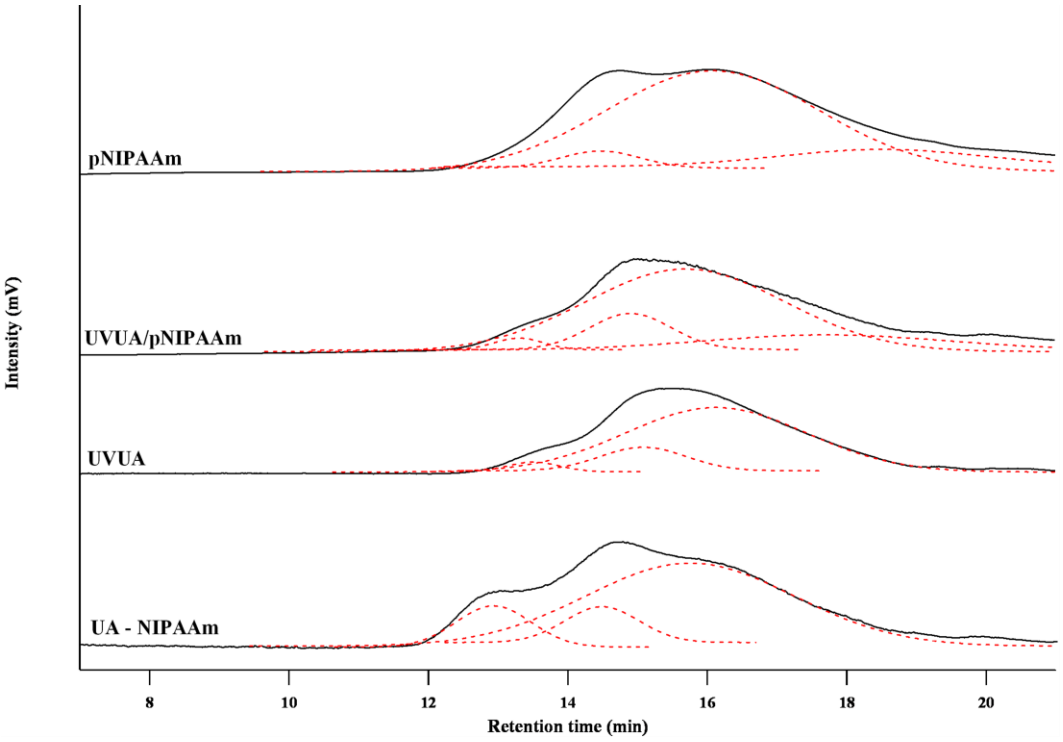

Fig. 8. GPC analysis of samples with relevant peak deconvolution.

Such distributions were ascribed both to the peculiar behaviour of ulvan in aqueous solution and the method of preparation of the polymers. Molar mass analysis of ulvan derivatives by GPC is commonly characterized by the presence of broad molecular weight distributions due to self-aggregation behaviour of the polysaccharide in aqueous solution (Robic, Gaillard, Sassi, Lerat & Lahaye, 2009). The widely spread molecular weight distribution of pNIPAAm and relevant copolymer was attributed to the selected method of polymerization of the monomer carried out under uncontrolled free radical conditions.

UA-NIPAAm was expected to elute faster as regards to its constituting homopolymers (UVUA and pNIPAAm) due to its higher molecular weight. The complex profile of the chromatograms recorded

during the analysis did not allow to clearly evidencing the expected outcome. The deconvolution of the peaks whose relevant areas are reported in Table 1 provided a better estimation of the molecular weight of the different populations constituting the analyzed polymeric material.

Table 1

Experimental details relevant to GPC analysis

Entry		Peak 1	Peak 2	Peak 3	Peak 4
pNIPAAm	**Mn**	190400	8415	1220	-
	Area %	6.4	83.1	10.5	-
UVUA/pNIPAAm	**Mn**	308840	116960	22060	2110
	Area %	2.7	13.7	76.5	7.1
UVUA	**Mn**	286560	94500	26778	-
	Area %	3.5	82.2	14.3	-
UA – NIPAAm	**Mn**	540300	178200	31470	-
	Area %	10.1	75.1	11.8	-

All the tested samples showed different populations associated to different peaks as resulted from the deconvolution of their chromatograms. Each peak was characterized by its own number average molecular weight (Mn) and the corresponding percentage of integrated area (Area %).

The deconvolution of the chromatogram of the physical mixture UVUA-pNIPAAm showed a distribution of population different from that obtained by the chromatogram of UA-NIPAAm indicating that UA-NIPAAm was not a physical mixture of the constituting homopolymers.

GPC analysis of UA-NIPAAm was compromised by troublesome filtration of the solution prior to injection that unavoidably led to partial loss of material. Nevertheless the mean Mn values of the different populations associated to different peaks in the chromatogram of UA-NIPAAm were higher compared to those of the precursors and the physical mixture. In particular the Mn value and the relevant area of the fraction with lower retention time was significantly higher (540300 g/mol, area = 10.1 %) than that of the UVUA precursor (286560 g/mol, area= 3.5%) and the physical mixture (308840 g/mol, area=2.7%) thus confirming the formation of the copolymer.

3.2 Thermogelling behaviour of poly (N-isopropylacrylamide)-g-ulvan (UA-NIPAAm)

3.2.1 Tilting Method

The occurring of gelling was macroscopically evaluated at different temperature (25 °C, 34 °C and 37 °C) by observing the flowing behaviour of the analyzed solutions subjected to a rapid inversion of the vial (Tilting method, Fig. 9). The test was carried out on solutions containing different concentration of UA-NIPAAm in order to assess the critical gelation concentration (CGC) of the copolymer, defined as the minimum copolymer concentration at which the gelation behaviour could be observed (Loh, Goh & Li, 2007). CGC can be determined by various means of which tilting method is the most common (Jeong, Kim, & Bae, 2002).

At 25 °C the solutions containing UA-NIPAAm appeared clear and not viscous. A rising of temperature increased the viscosity and opalescence of the solutions. At 34 °C the tilting method indicated the formation of a weak gel in the case of solutions containing UA-NIPAAm with concentration higher than 3 wt%, while the formation of a stable gel was observed at 37 °C (Fig. 9).

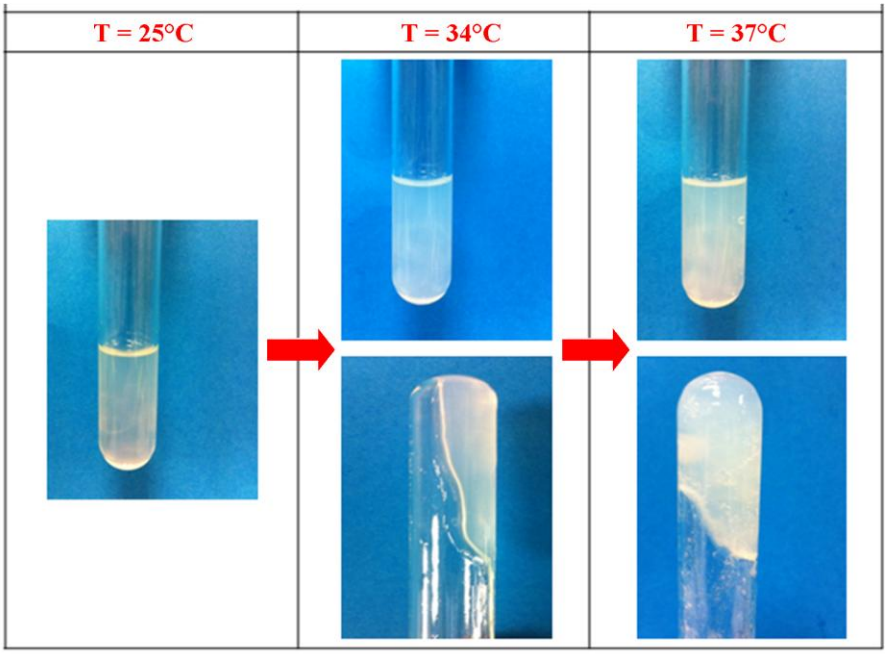

Fig. 9. Thermogelling behaviour of UA-NIPAAm solution (4 wt%, PBS 10 mM, pH 7,4) tested by tilting method at different temperatures.

Interestingly UA-NIPAAm gels showed no significant syneresis as compared to that encountered by using pure PNIPAAm thus representing a promising step toward the improvement of this material (Gan, Guan & Zhang, 2010)

The critical gelation concentration of UA-NIPAAm was found to range between 4 to 5 wt% whose values are typical of polymeric materials displaying very low CGC (Loh, Goh & Li, 2007). The ulvan

contribution to the recorded low CGC values was decisive due to its ability to hold large amounts of water (Morelli & Chiellini, 2010). This allows for the development of effective thermogelling systems by using small quantities of polymeric materials, thus reducing toxicity concerns and operational costs.

3.2.2 Thermal analysis by DSC: phase transition behaviour (LCST)

The thermogelling behaviour of UA-NIPAAm in aqueous solution stems from the phase transition of the copolymer from soluble to insoluble, provided by the grafted pNIPAAm chains. DSC analysis was selected as reliable method to objectively determine the temperature of the occurring transition (.Li, Shan, Yue, Lam, Tam & Hu, 2002; Liu, Shao, & Lu, 2006). All the recorded DSC traces showed an endothermic first order transition temperature whose minimum was taken as LCST value of the copolymer at the specified conditions. The analysis was carried out on a set of solutions having different UA-NIPAAm concentrations ranging from 2 wt% to 5 wt% in order to assess the behaviour of the copolymer below and above its CGC value. PBS (10 mM, pH = 7.4) was selected as optimal medium to simulate the physiological environment that could be experienced by the developed injectable system in the final applications.

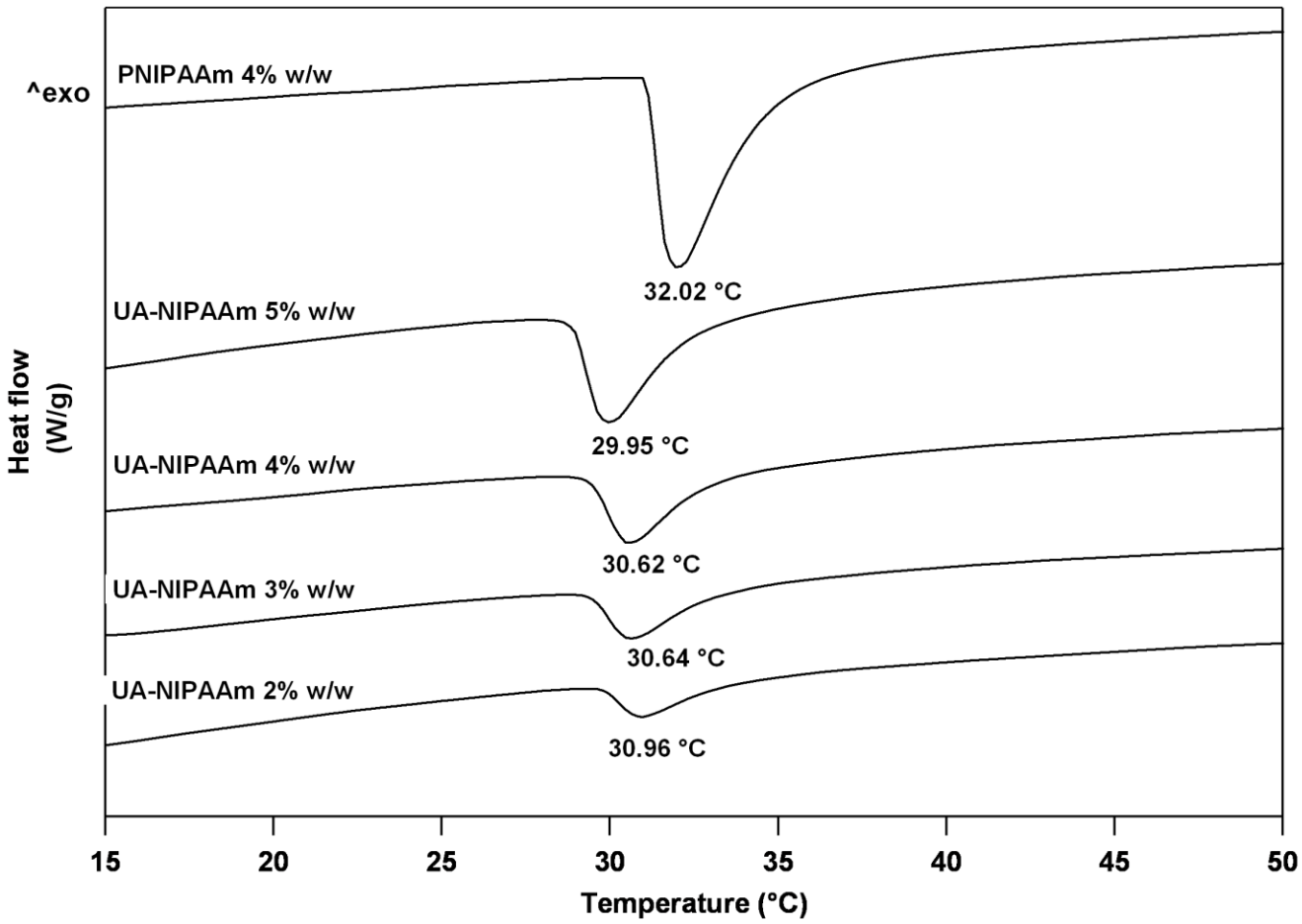

Fig. 10. Comparison of the DSC traces of UA-NIPAAm samples dissolved in PBS (10 mM, pH = 7.4) at different concentrations.

The LCST values recorded for the different UA-NIPAAm solutions were found to be close to that of PNIPAAm indicating a negligible effect of Ulvan in the modification of the thermogelling behaviour of PNIPAAm (Fig. 10). The LCST value of PNIPAAm constituting grafted and block copolymers are known to be altered by the chemical nature of the comonomers according to their hydrophilicity or hydrophobicity (Ohya, Nakayama & Matsuda, 2001) (Chen & Cheng, 2006). Hydrophilic components were demonstrated to increase the LCST values of pNIPAAm by preventing the dehydration of the polymeric chains (Tan, Ramirez, Miljkovic, Li, Rubin & Marra, 2009) whereas the introduction of hydrophobic components may decrease the LCST of pNIPAAm, due to the easing of the dehydration of copolymer chains (Lian, Xiao, Bian, Xia, Guo, Wang & Lang, 2012). The LCST values of UA-NIPAAm could be interpreted by considering the amphiphilic behaviour of ulvan provided by the hydrophilicity of sulphate, carboxylic and hydroxyl groups and the hydrophobicity provided by the

modification of hydroxyl with acrylate groups and the large presence of methyl groups of rhamnose residues. Indeed the methyl groups of rhamnose have been considered as being responsible for the unusual hydrophobic behaviour of ulvan despite its polyelectrolyte nature (Robic, Gaillard, Sassi, Lerat & Lahaye, 2009)..

The LCST values recorded for the different UA-NIPAAm solutions were found to be not substantially affected by the copolymer concentrations as well although a slight decrease was evidenced in the more concentrated solutions. The whole range of the analyzed concentrations proved to be potentially suitable for the development of thermogelling systems since the recorded LCST values were significantly lower than the basal body temperature of living organisms. Nevertheless the outcomes of the Tilting Method test revealed that exclusively the more concentrated solutions were able to provide stable thermogelation. This behaviour could be interpreted by considering that DSC analysis effectively recorded the thermodynamic transition irrespective of the concentration of the analyzed solution as long as the recorded heat is within the limit of instrumental sensitivity. The macroscopic effects of the phase transition were evidenced in the more concentrated solutions since both overlapping and entanglement of polymeric chains are required for observing gelation.

3.2.3 Rheological studies

The rheological behaviour of the 4% wt% UA-NIPAAm solution in PBS (10 mM, pH = 7.4) was studied to corroborate the results obtained by DSC analysis and tilting method about the thermogelling ability of the developed material. The analysis was carried out by means of a parallel plates rheometer in the temperature range of 15 to 40 °C and at a shear stress of 2 Pa, whose value was found to lie within the linear viscoelastic regime. The data were collected at fixed temperatures (15°C, 20°C, 25°C, 30°C, 35°C, 37°C, 40°C) to collect more accurate results not affected by the heating speed and to better simulate the operative conditions. The shear viscosity η and the complex shear modulus G* of the solutions calculated at the selected temperatures, expressed as mean ± standard deviation from 3 samples, are reported in Figure 11.

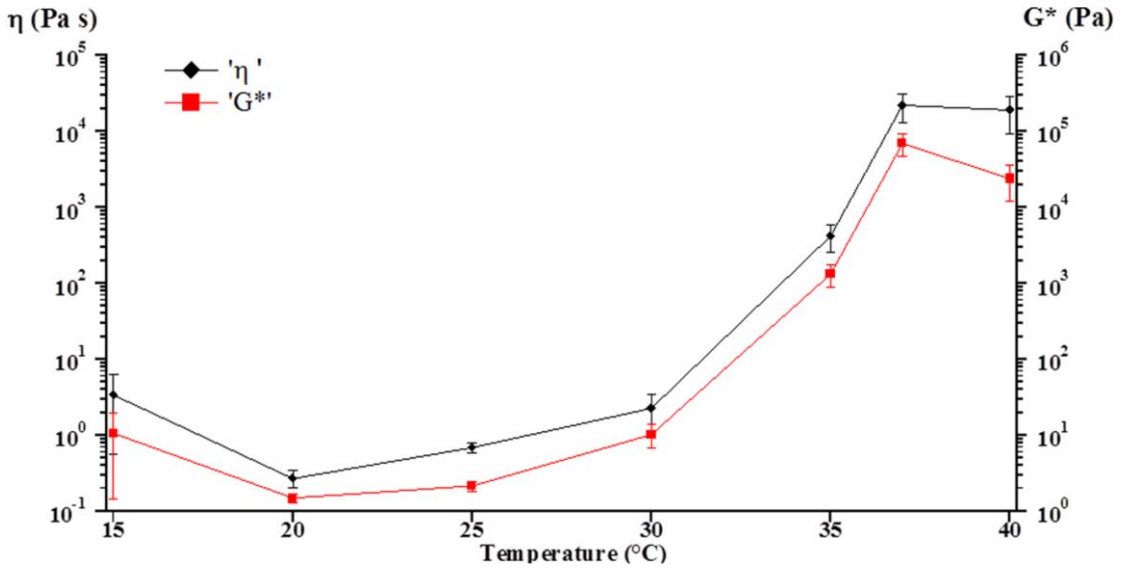

Fig. 11. Shear viscosity η and complex shear modulus G* of 4% wt% UA-NIPAAm solution in PBS (10 mM, pH = 7.4) recorded at different temperatures.

Both the shear viscosity and the complex shear modulus of the UA-NIPAAm copolymer solutions increased drastically in the range 30 – 37 °C indicating a transition from a liquid-like behaviour to an elastic gel-like behaviour. Above 37 °C, η and G* levelled off, indicating that the network structure of the hydrogels was completely formed thus objectively corroborating the results obtained by Tilting Method.

4. Conclusions

The exploitation of waste biomass is nowadays arousing growing interest among the scientific community since it allows to capitalize sustainable and renewable resources of energy and materials while reducing stringent environmental concerns. The work described in the present paper represents an effective example pursuing this strategy, since it reports the successful conversion of algal biomass whose fate is accumulation and uncontrolled degradation over landfill and seashores, into high value added materials such as those for biomedical applications. To the best of our knowledge the present study demonstrates, for the first fime, the feasibility of producing thermosensitive injectable hydrogels based on ulvan, a underexploited sulphated polysaccharide of algal origins with peculiar chemical properties not easily found in other natural polymers. The rheological properties and thermal behaviour of the developed materials revealed suitable to match those necessary to in situ gelling systems for biomedical applications. The processes implemented in this work were designed to limit environmental issues through the use of biocompatible and bio-degradable/eliminable materials, safe

solvents and mild experimental conditions. Straightforward and clean procedures were adopted in view of an easy reproduction on larger scale. The whole process represents a solid base for the development of smart stimuli responsive hydrogels through the exploitation of renewable and sustainable resources.

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Figure 1

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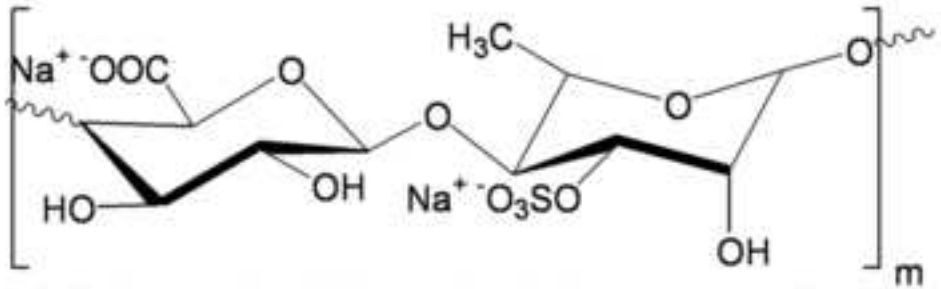
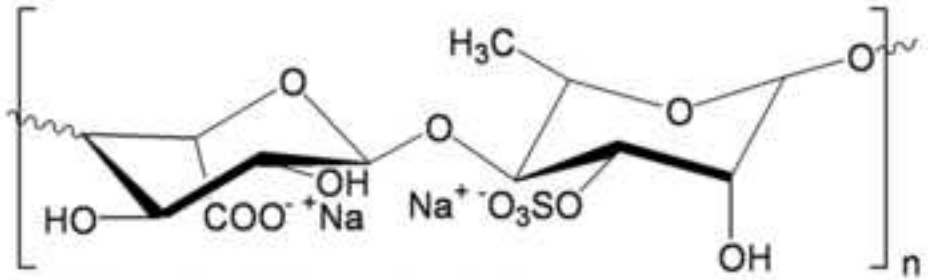


β-D-glucuronic acid (1→4)-α-L-rhamnose-3-sulfate



α-L-iduronic acid (1→4)-α-L-rhamnose-3-sulfate

Figure 2

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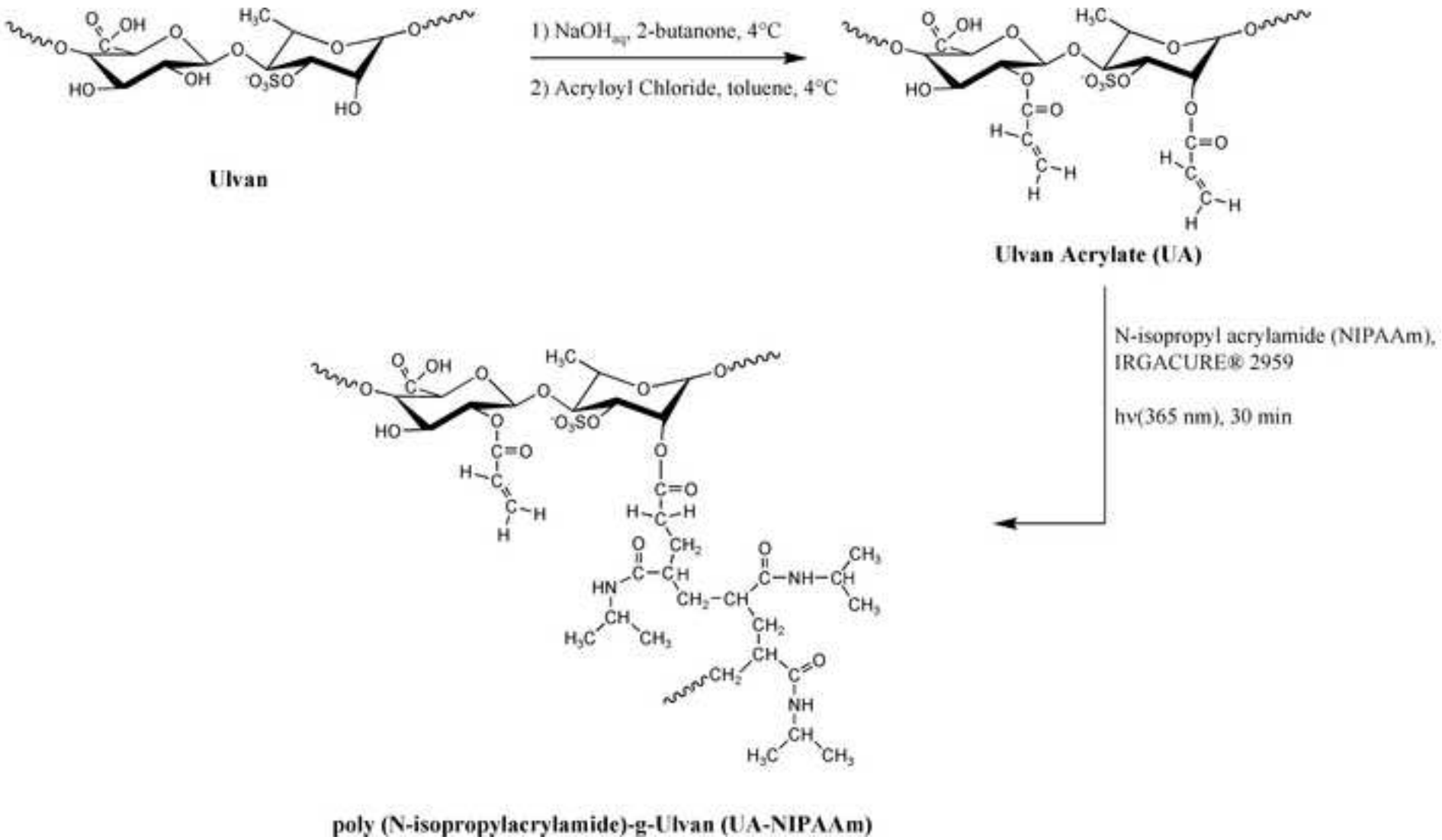

Ulvan

Ulvan Acrylate (UA)

poly (N-isopropylacrylamide)-g-Ulvan (UA-NIPAAm)

Figure 3

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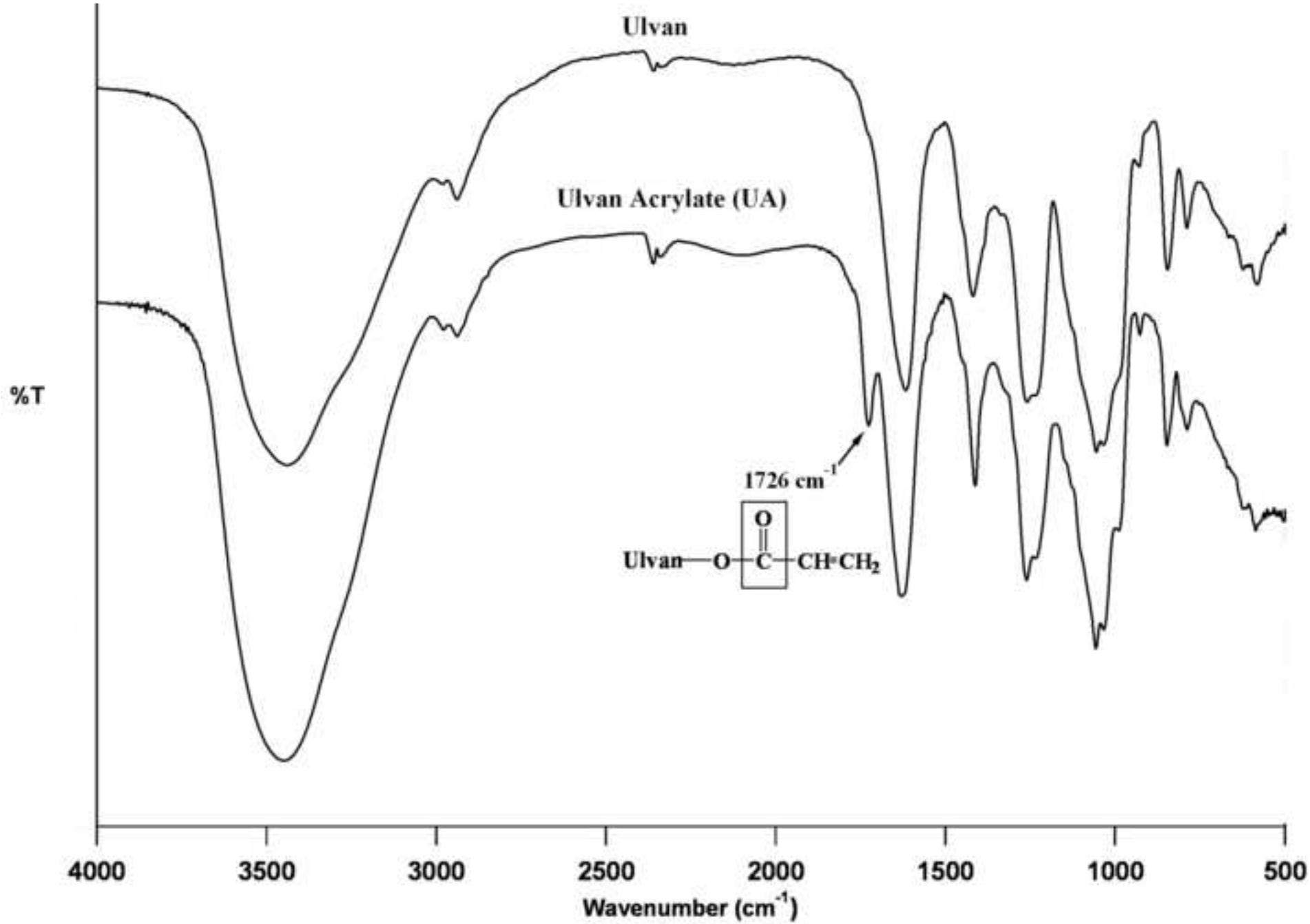

Figure 4

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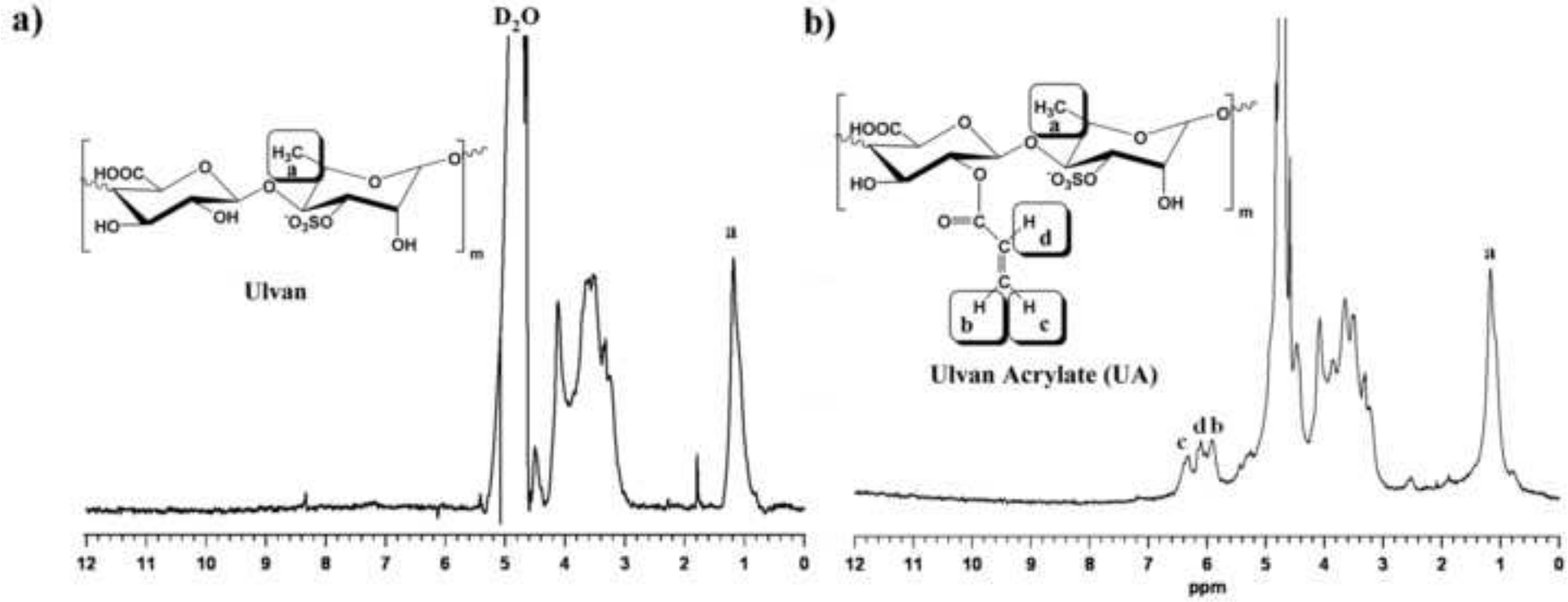

Figure 5

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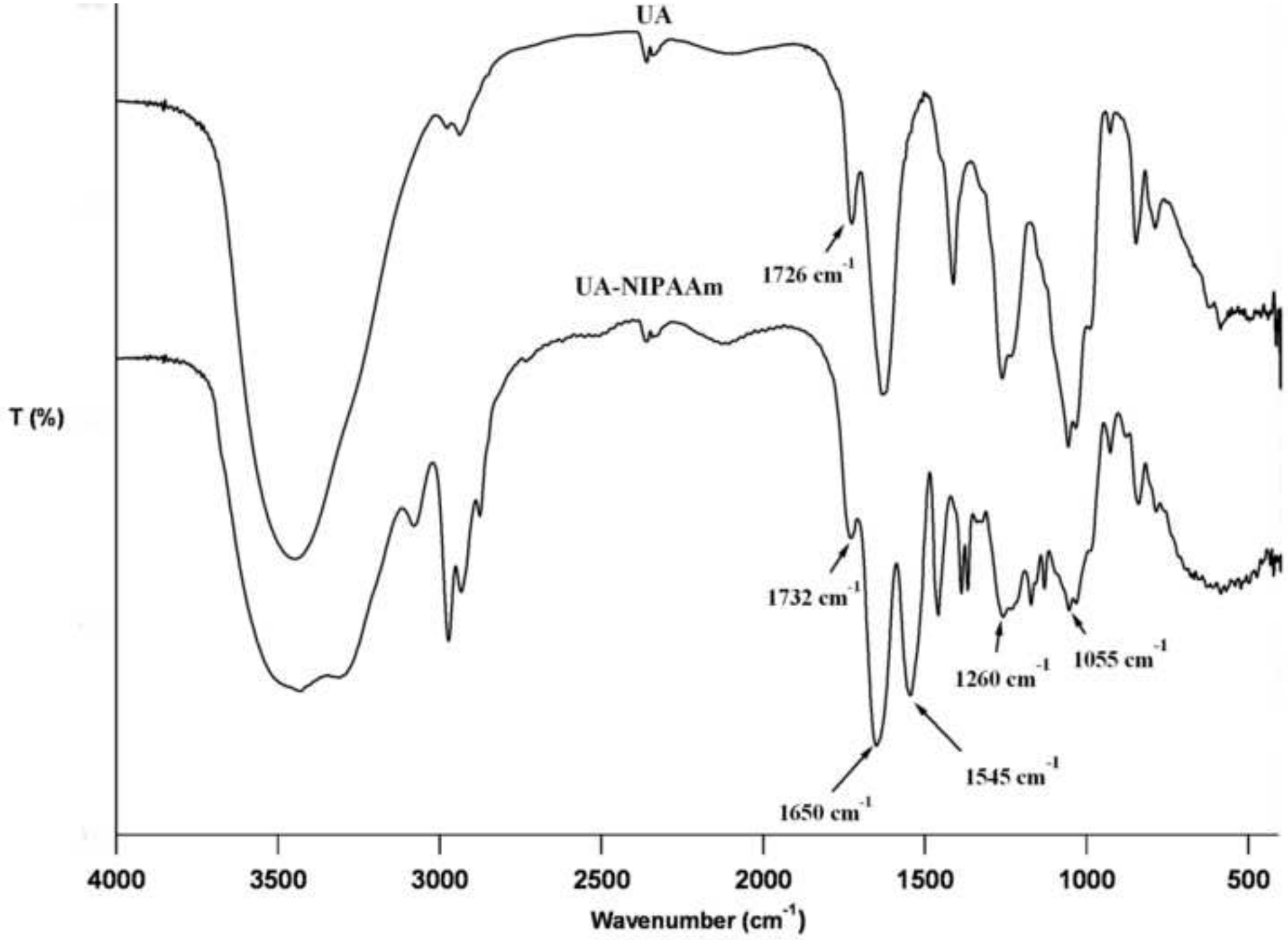

Figure 6

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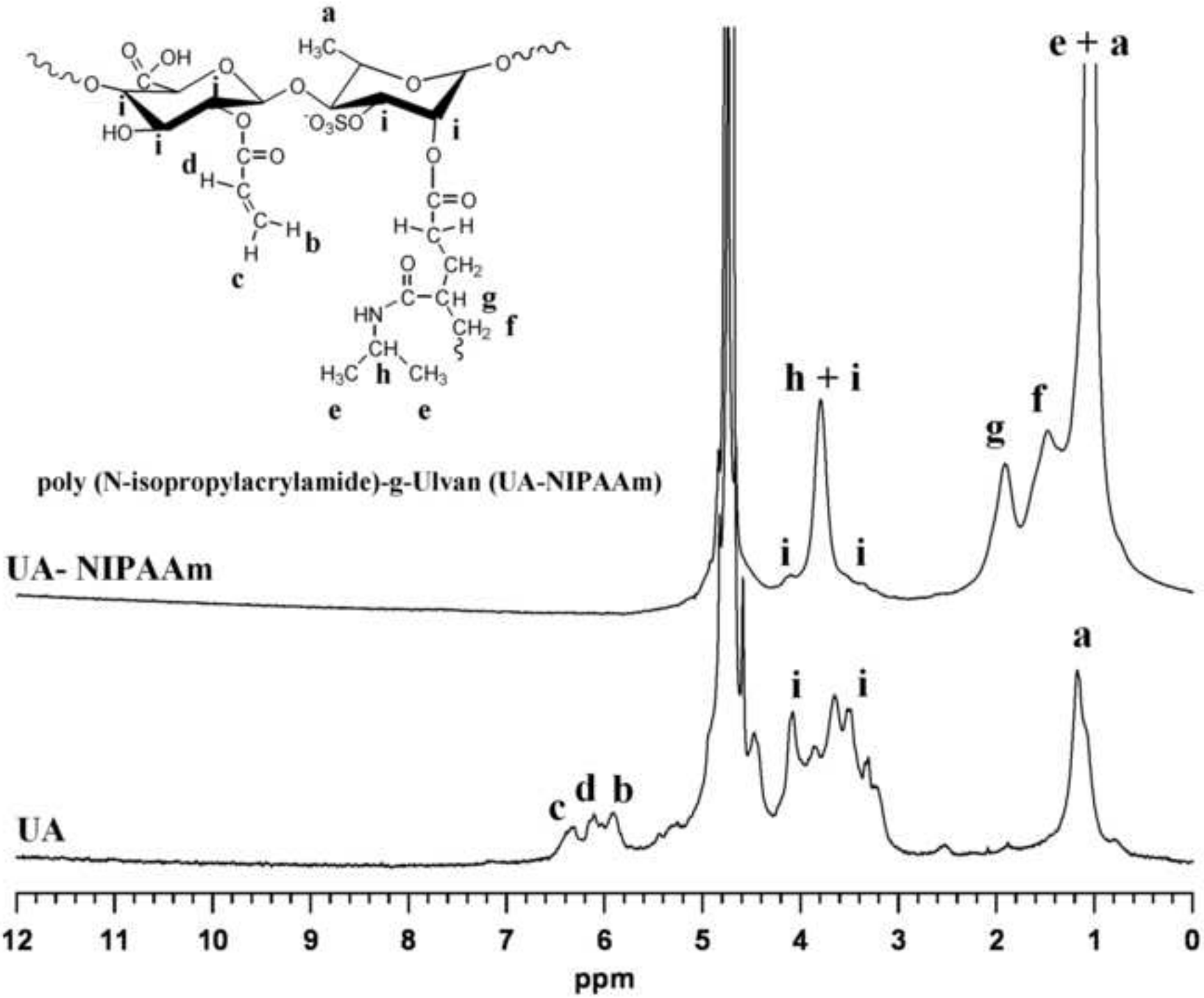

Figure 7

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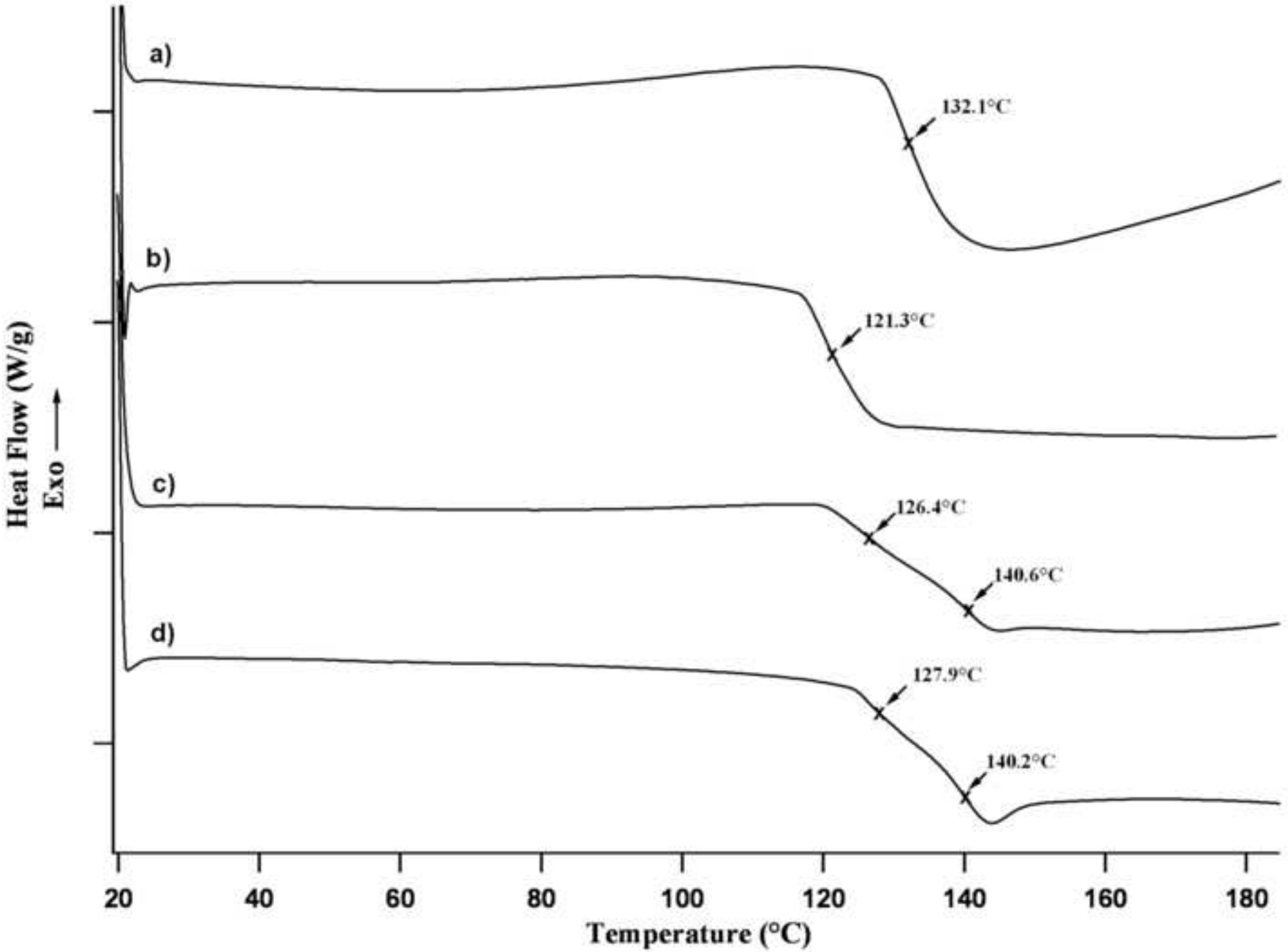

Figure 8

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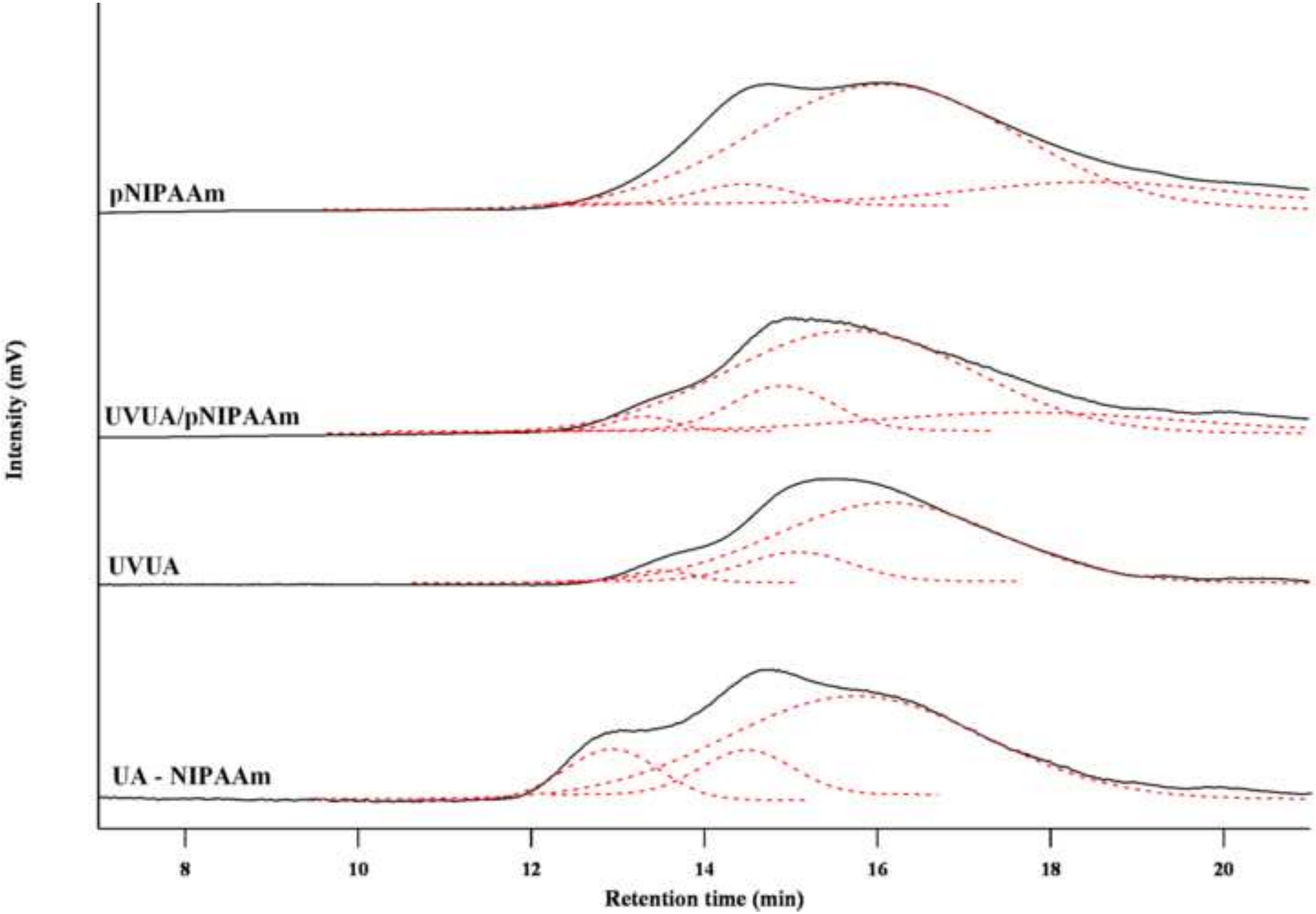

Figure 9

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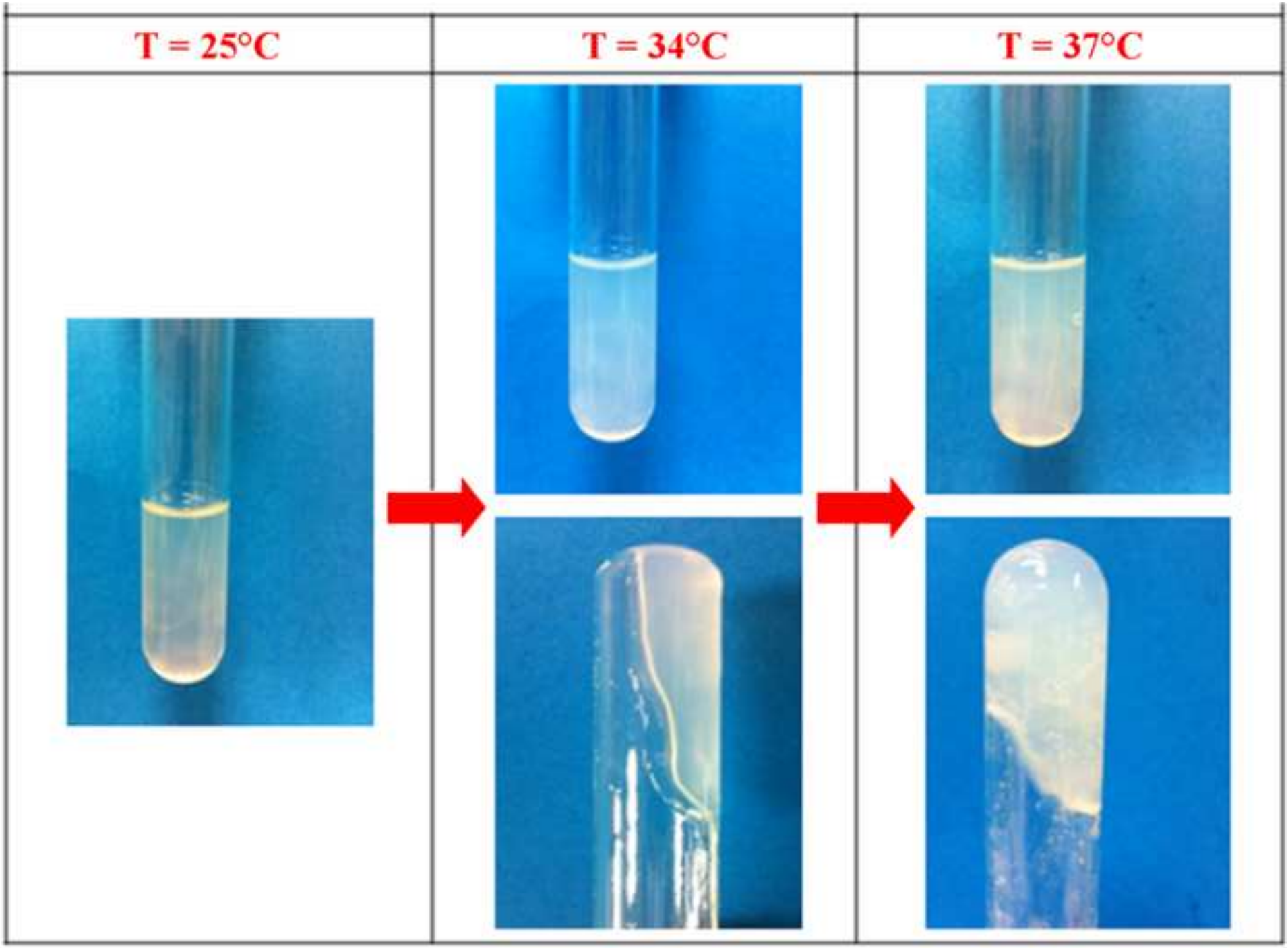

Figure 10

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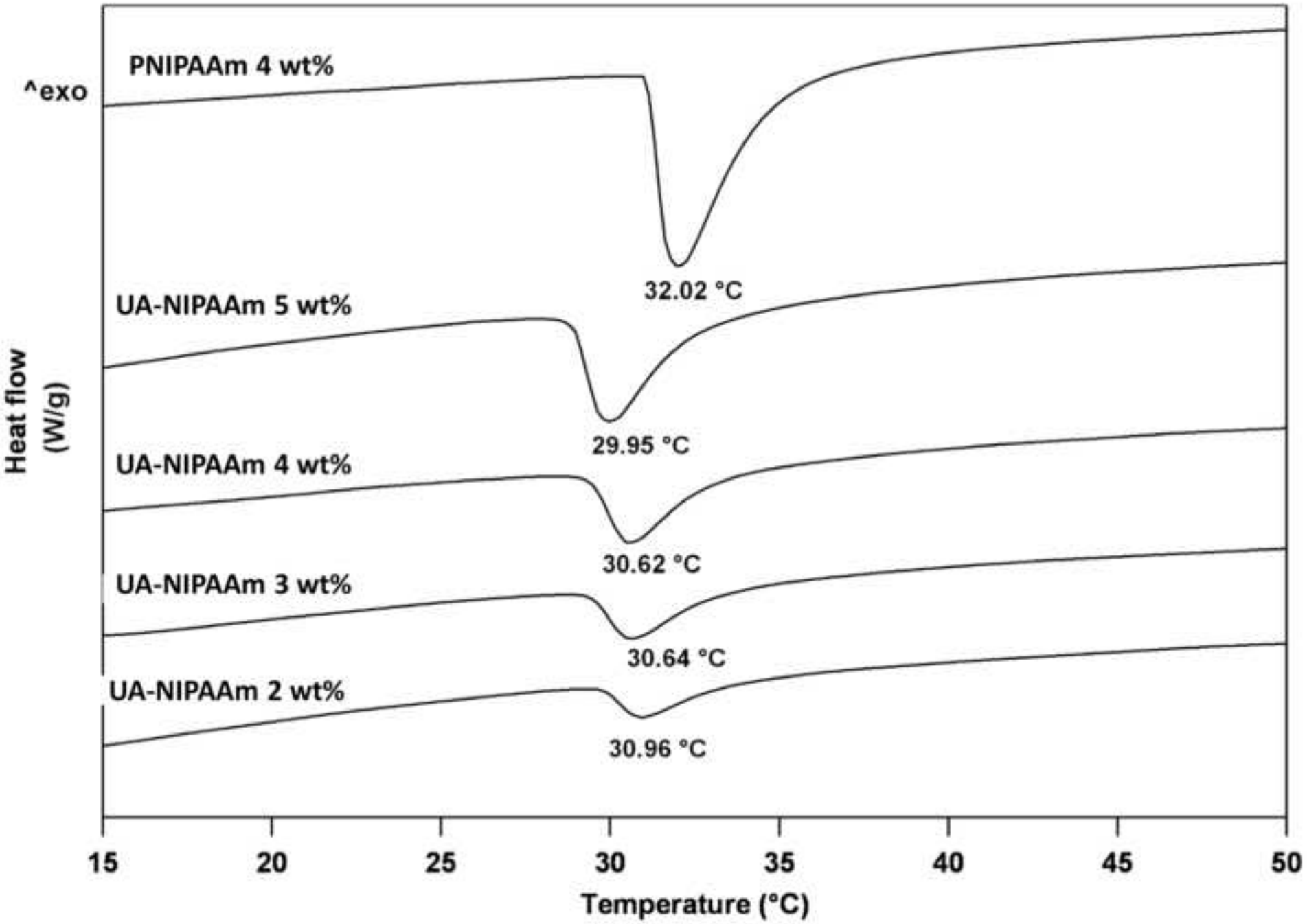

Figure 11

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