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Full Paper

Design, preparation and characterization of thermoresponsive hybrid nanogels using a novel ulvan-acrylate crosslinker as potential carriers for protein encapsulation.

Andrea Morelli, Dario Puppi, Victoria Cheptene, Dunia Disgraziati, Giacomo Ruggeri, Federica Chiellini*

Dr. A. Morelli, Dr. D. Puppi, V. Cheptene, D. Disgraziati, Prof. F. Chiellini
BIOLab Research Group, Department of Chemistry and Industrial Chemistry, University of Pisa, UdR-INSTM PISA via Moruzzi 13, 56124, Pisa, Italy
E-mail: federica.chiellini@unipi.it
Prof. G. Ruggeri
Department of Chemistry and Industrial Chemistry, University of Pisa, UdR INSTM Pisa, via Moruzzi 13, 56124, Pisa, Italy

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Abstract: The aim of the present study was the design and development of thermoresponsive nanogels based on ulvan, a sulphated hetero-polysaccharide of algal origins with unique biological and chemical properties. Hybrid nanogels were successfully synthesized by means of UV initiated radical copolymerization of N-vinylcaprolactam (NVCL) with an ulvan derivate (UA) as a novel crosslinker. In nanogels the ulvan-grafted poly(N-vinylcaprolactam) (PNVCL) chains represent the thermo-responsive component. The most promising candidates, selected after a thorough physical-chemical characterization of nanogels in terms of size and responsivity to thermal variation at physiological conditions, were loaded with bovine serum albumin (BSA) as model bioactive compound. The developed nanogels displayed BAS loading efficiency values similar to those obtained by using synthetic crosslinkers, and thus

indicating the suitability of the developed ulvan-acrylate to act as novel macromolecular crosslinker for thermoresponsive nanogels preparation.

1. Introduction

The compelling need to develop more performing polymeric devices for biomedical applications led the research to investigate novel aspects related to the possible exploitation of the topological and dimensional properties of the polymers.[1] To this aim scientific attention has been increasingly focused on the development of smart polymers due to their unique ability to respond with conformational changes to external stimuli such as pH, temperature and salinity.[2] The conformational fluctuations occurring in smart polymers on molecular scale ultimately produce macroscopic topological and dimensional variations in their relevant devices, which could be suitably exploited for crucial applications. In this scenario, thermoresponsive polymers which are known to undergo a phase transition from liquid to gel below the lower critical solution temperature (LCST) represent a very promising class of materials.[3] Their significant conformational change, ranging from an extended hydrophilic state to a collapsed hydrophobic state occurring during the phase transition, can be suitably exploited for the controlled release of loaded cargos.[4] Indeed, thermoresponsive polymers have been increasingly applied in biomedical applications for the development of smart macromolecular devices such injectable hydrogels and micro/nanogels for the targeted release of cells and therapeutic agents.[5-6] The proper selection and optimization of the properties of the thermoresponsive matrix, whose LCST value must be modulated close to physiological temperature, allows to targeting the release of the loaded cargos into the desired site of action hence drastically reducing the invasiveness and negative effects of traditional devices.[5-6] Among smart polymeric materials, thermoresponsive nanogels have been increasingly investigated since they combine the advantages of nanoparticles and hydrogels in a single device.[7] As highly swollen three-dimensional matrices they can be loaded with a large variety of therapeutic agents such as drugs, proteins and peptides which can be ultimately released in a very efficient manner due to the large surface exposed to the external environment as nano-sized materials.

Thermoresponsive nanogels, which respond with suitable physical change to thermal stimuli have been increasingly investigated as delivery system in cancer therapy since they efficiently allow for the targeted release of therapeutic agents in pathological sites.[6] Among the thermoresponsive polymers used for drug delivery applications poly (N-isopropylacrylamide) (PNIPA) certainly represented the most investigated material since it showed an LCST value above physiological temperature and fair biocompatibility.[8] However alternative thermoresponsive polymers were recently investigated since PNIPA was lately reported to degrade into toxic amine compounds under acidic conditions.[9-11] Poly (vinylcaprolactam) (PNVCL) has been investigated as promising thermoresponsive polymer alternative to PNIPA since it undergoes to phase transition below physiological temperature and does not release toxic compounds in the environment under acidic conditions.[9-11] Acidic hydrolysis which provoke the release of toxic amines from PNIPA do not affect the healthy profile of PNVCL since the hydrolyzed amide group is hold linked to the polymeric backbone as part of the lactam ring (Figure 1a).

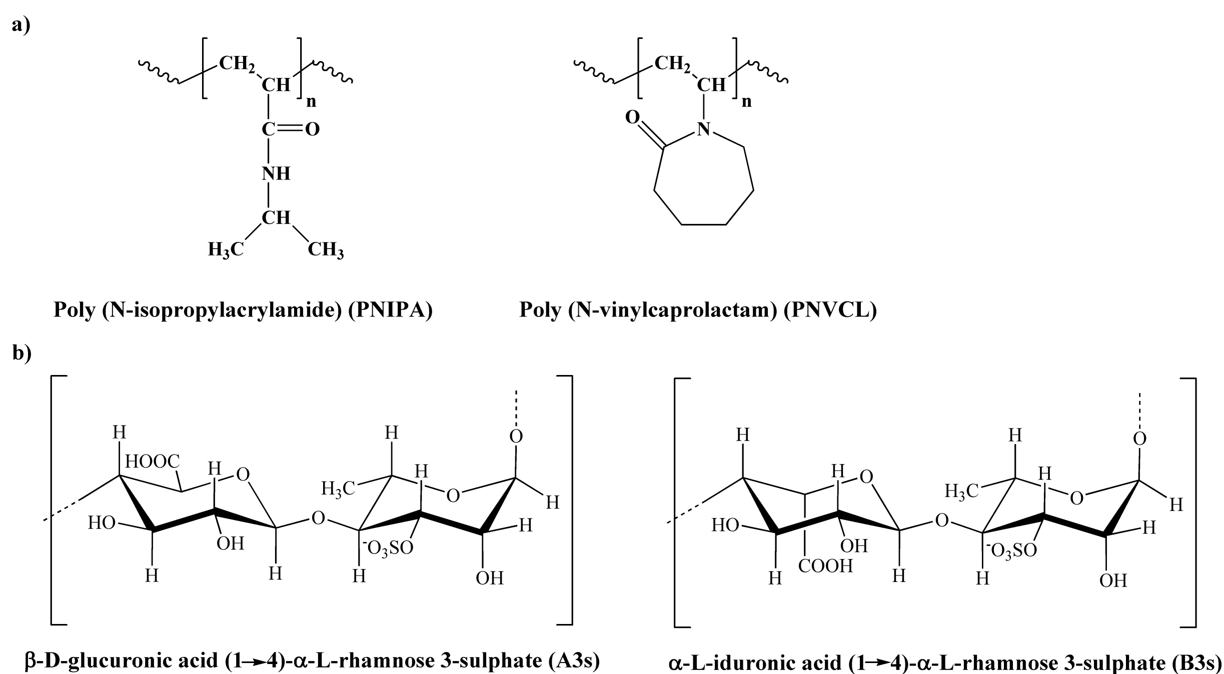

Figure 1 Chemical structure of the repeating units of a) PNIPA and PNVCL and b) the main repeating units of ulvan constituted by a disaccharide sequence of aldobiuronic acid compounds

The use of PNVCL as thermoresponsive component for the fabrication of smart nanogels is relatively recent and mainly based on the use either of synthetic crosslinkers such as N,N-methylenebisacrylamide or comonomers of petrochemical origins such as substituted methacrylates and methacylic acid.[12-15] To the best of our knowledge only few papers were reported in literature describing the use of natural crosslinkers for the synthesis of nanogels.[16-17] Aguirre et al. reported the development of PNVCL-based nanogels using polysaccharides as macromolecular crosslinkers.[16] The use of dextran, suitably modified with methacrylate groups, accounted for the synthesis of natural-derived nanogels with improved biocompatibility, biodegradability and morphology than those obtained with synthetic crosslinkers.[16] Our aim was to investigate the feasibility of using ulvan, a sulphated hetero-polysaccharide extracted from green algae belonging to Ulva sp., as a natural macromolecular crosslinker for the synthesis of thermoresponsive nanogels (Figure 1b). Ulvan represents an underexploited material with unique chemical versatility and bioactivity that is recently emerging as a promising candidate for biomedical applications.[18-19]

It is especially attracting due to its inherent biocompatibility and tremendous availability from abundant and renewable resources whose exploitation could help to remediate environmental concerns and contribute to converting waste materials into high-value added devices.[18-19] Ulvan could represent a promising candidate as macromolecular crosslinker for nanogel fabrication thanks to its high water uptake capability, which is useful to provide the thermoresponsive devices with enhanced volume variation during the phase transition.[20] To date the investigations of ulvan in biomedical areas have been limited to works mainly aiming at the fabrication of implantable and injectable hydrogels.[20-23] In the present paper we report the unprecedented preparation of thermoresponsive nanogels based on an ulvan derivate as macro-crosslinker and PNVCL as thermo-responsive component. A straightforward and rapid method was used to fabricate the nanogels by radical copolymerization of N-vinylcaprolactam monomer (NVCL) onto suitably modified ulvan with acrylate groups mediated by UV

irradiation. The most promising candidates that emerged from a thorough physicochemical characterization were investigated as potential carriers for protein encapsulation by using BSA as model compound.

2. Experimental Section

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-vinylcaprolactam (NVCL) (> 98%, Sigma-Aldrich) was distilled under vacuum before use. Acryloyl chloride (AC) (> 97%), 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (IRGACURE®2959) (98%) and bovine serum albumin (BSA) (> 96%) were purchased from Sigma Aldrich. Poly (N-vinylcaprolactam) (PNVCL) (Mn = 10900, Mw/Mn = 1.30) was synthesized according to a procedure reported by Liang et al..[24] Phosphate Buffer Saline (PBS) 0.01 M, pH 7.4 was prepared by dissolving 8.0 g of NaCl, 0.2 g of KH_2PO_4, 0.2 g of KCl 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 HCl 1N and the resulting solution was steam sterilized (121°C for 20 min) before use and storage. Unless otherwise specified, reagents were used as received without further purification.

2.2 Synthesis

2.2.1 Preparation of ulvan-acrylate (UA)

Ulvan-acrylate conjugate (UA) was synthesized according to a procedure reported by the literature by Morelli et al..[23] In a 100 ml round bottomed, three-necked flask equipped with a magnetic stirrer and dropping funnel, 1.0 g of ulvan (2.5 mmol of disaccharide repeating units corresponding to 7.5 mmol of reactive hydroxyl groups) was firstly added to 20 ml of

deionized water. The obtained dispersion was kept stirring overnight at room temperature to allow for the complete dissolution of the polysaccharide. 2-butanone (4 ml) and an aqueous solution containing NaOH (8 mL) were sequentially added to the obtained mixture and the resulting solution was kept stirring for 30 minutes at 4°C to allow for the activation of the hydroxyl groups of ulvan. Two different experiments were carried out by varying NaOH concentration (**Table 1**). Finally a large excess of acryloyl chloride (16 ml; 198 mmol; 26:1 molar ratio of AC to reactive Ulvan hydroxyl groups) in toluene (20 ml) was added dropwise to the reaction mixture at 4 °C and the obtained solution was kept under magnetic stirring for 4 h in the same conditions.

Table 1. Experimental details relevant to chemical modification of ulvan with AC

Ulvan acrylate	**$[AC] / [\text{-}OH]_{ulvan}$**	**$[NaOH] / [\text{-}OH]_{ulvan}$**	**SD^a**
UA-1	26.0	30.0	0.6
UA-2	26.0	15.0	0.2

a: Substitution degree was measured by ^{1}HNMR as the mean number of acryloyl group present in each repeating unit of UA.

The aqueous phase containing the modified ulvan was separated from the organic phase by means of a separatory funnel and added dropwise to a 250 mL flask containing absolute ethanol (1:10 v/v) in order to purify the polysaccharide by precipitation. After centrifugation the product was repeatedly washed with absolute ethanol and then dried under vacuum. The obtained solid was further purified by exhaustive dialysis against deionized water (Cellulose Ester, MWCO = 10000, Spectra/Por® Biotech) for 48 hours and freeze-dried at -50 °C. Typical product yields ranged from 60 to 75 %.

2.2.2 Preparation of ulvan-g-poly(N-vinylcaprolactam) based nanogels (UA-PNVCL)

Solutions at different concentration of photoinitiator (IRGACURE® 2959), UA macromer and NVCL monomer 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 $mW·cm^{-2}$, Helios

Italquartz) for 15 minutes under magnetic stirring at the temperature provided by the heating effect of the irradiating high pressure UV lamp (70° C). The amounts of the reagents used in each experiment are reported in Table 2.

The obtained dispersions were then purified by exhaustive dialysis against water (Cellulose Ester, MWCO = 10000, Spectra/Por® Biotech) for 48 hours and freeze dried at -50 °C. The dialysis step of the blank experiment was carried out by using a different membrane (Cellulose Ester, MWCO = 3500, Spectra/Por® Biotech).

Table 2. Experimental details relevant to UA-NVCL-based nanogels preparation.

Entry	UA (w/v %)	NVCL (w/v %)	NVCL/UA[a] (w/w)	Irgacure ® 2959 (w/v %)	NVCL conversion[b] (%)	NVCL/UA[b] (w/w)	UV irradiation (min)
UA-1-1	0.2	4.0	20.0	0.005	35	7.1	15
UA-1-2	0.1	4.0	40.0	0.005	n.a[c]	n.a.[c]	15
UA-2-1	0.2	4.0	20.0	0.005	60	11.8	15
UA-2-2	0.1	4.0	40.0	0.005	22	8.9	15
Blank	-	4.0	-	0.005	0	0	15

a: Polymerization feed ratio.
b: Referred to the fraction of monomer grafted onto UA calculated by postulating that all UA used in the polymerization feeding ratio was completely recovered in the final product purified by dialysis.
c: not available since macrogelation occurred.

2.3 Protein encapsulation

2.3.1 Loading of BSA onto UA-PNVCL nanogels

A solution of BSA (0.150 mL, 1mg/mL) in PBS (0.01 M, pH 7.4) was added to a UA-PVNCL-based nanogel dispersion (10 mg/mL) in PBS (0.01 M, pH 7.4). The obtained mixture was incubated at 4°C for 24 hours under gentle magnetic stirring. After centrifugation (14000 rpm, 15 min) the supernatant liquid was submitted to bicinchoninic assay (BCA assay) in order to determine the amount of BSA loaded onto the nanogels (Paragraph 2.3.2). Blank experiments were carried out separately in order to assess the contribution to the BCA assay either of the nanogel dispersions in absence of BSA and the protein in absence of nanogels.

2.3.2 Protein encapsulation

Protein encapsulation was determined by using a Micro BCA™ protein assay kit (ThermoFisher Scientific, Wilmington, DE). Plate reading was carried out on a Bio-Rad Benchmark Microplate Reader (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. The amount of protein loaded onto UA-PNVCL nanogels was determined by difference by using **Equation 2.1**:

$$Protein\ loading\ =\ total\ protein - free\ protein \qquad (Equation\ 2.1)$$

where free protein was the amount of unloaded protein detected in the supernatant liquid of the nanogel mixture whose value was obtained by interpolating the absorbance recorded at 565 nm by BCA analysis with a calibration curve obtained by using BSA as standard (1-200 µg/mL). Loading efficiency % was calculated according to **Equation 2.2**:

$$Loading\ efficiency\ \%\ =\ \frac{Protein\ loading}{Total\ protein} \times 100 \qquad (Equation\ 2.2)$$

2.4 Characterization

2.4.1 FT-IR analysis

FT-IR spectra were recorded on KBr pellets (2% w/w) in the range of 4000–400 cm^{-1} by using a Jasco FT-IR 410 spectrophotometer. Each spectrum was recorded after 32 scans by using a resolution of 4 cm^{-1}.

2.4.2 ^{1}HNMR analysis

NMR spectra were recorded by using a Varian Gemini 200 spectrometer interfaced with a Sparc4 (Sun) console and VNMR6.1B software. The analyses were carried out on 2% (w/v)

solutions of samples dissolved in deuterium oxide. Spectra were processed by using SpinWorks software (version 3.1.7.0).

2.4.3 DSC analysis

2.4.3.1 Determination of thermal properties

DSC analysis was carried out on conditioned samples (5-10 mg) by using a Mettler DSC-822 (Mettler Toledo, Milan, Italy) under an 80 ml/min nitrogen flow. Sample conditioning were performed by drying the materials under vacuum at 70°C for 1 hour. Each experiment was composed by two heating cycles spaced out by a cooling step. Accordingly 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 temperature (Tg) was taken as the inflection point in the second heating cycle thermogram.

2.4.3.2 Determination of the Lower Critical Solution Temperature (LCST)

Nanogel dispersions (4 wt%) in PBS (0.01 M, pH = 7.4) were introduced into hermetically sealed standard aluminum crucibles and submitted to DSC analysis to determine their LCST values. DSC thermograms were recorded in the temperature range from 10 to 60°C at a heating rate of 10°C/min. The LCST value was considered as the minimum of the endothermic peak of the recorded curve.

2.4.4 Dynamic light scattering analysis (DLS)

Dynamic light scattering (DLS) measurements were conducted by using a Delsa Nano C from Beckman Coulter, Inc. (Fullerton, CA) equipped with a laser diode operating at 658 nm. Scattered light was detected at 165° angle and analyzed by using a log correlator over 30 accumulations for a 2.0 mL of sample in a glass size cell. Nanogel dispersions (0.5 wt%) in

PBS (0.01 M, pH = 7.4) were equilibrated at each selected temperature for 10 minutes before analysis. Particle size distribution and distribution averages were calculated by using CONTIN particle size distribution analysis routines through Delsa Nano 3.73 software. The hydrodynamic radius and polydispersity index of the analyzed dispersions were calculated on three replicates of each sample by using the cumulant method.

3. Results and Discussion

The strategy adopted for the preparation of ulvan-based thermoresponsive nanogels envisaged the grafting copolymerization of NVCL onto the polysaccharide side chains by means of radical process promoted by UV irradiation. To this aim ulvan was suitably modified with alkenyl groups in order to act both as as grafting-from macromer during the polymerization of NVCL and as crosslinker of the growing thermoresponsive PNVCL polymeric chains (Figure 2).

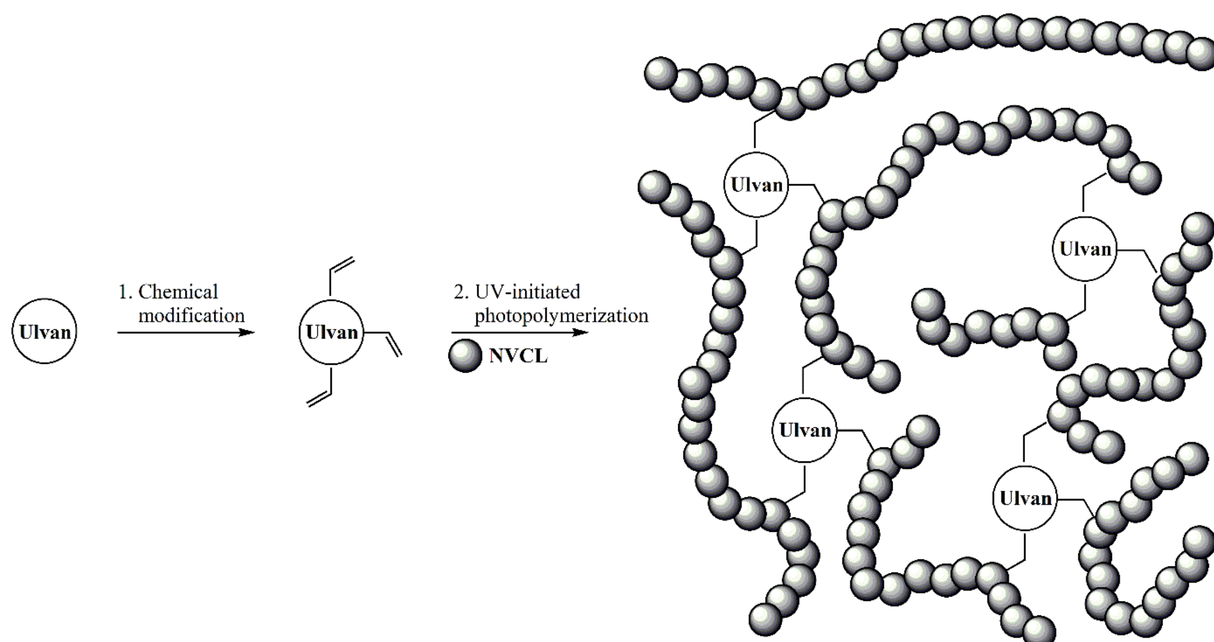

Figure 2. Schematic representation of thermoresponsive poly (N-vinylcaprolactam)-*g*-Ulvan (UA-PNVCL) based nanogels preparation.

3.1 Preparation of poly (N-vinylcaprolactam)-g-Ulvan (UA-PNVCL) based nanogels

3.1.1 Synthesis and characterization of Ulvan-Acrylate (UA)

Ulvan was submitted to chemical modification with alkenyl groups in order to be susceptible to radical polymerization according to a synthetic procedure reported by our group.[23]

Acrylate group was selected as alkenyl-bearing functionality due to its inherent large reactivity in radical polymerization provided by electronic and steric effects. Acryloyl functionalities were conjugated to the polysaccharide backbone as carboxylic ester groups to make the crosslinked material sensitive to hydrolytic degradation in physiological conditions. Hydroxyl groups of ulvan were esterified by using AC as alkenyl group precursor in virtue of the high reactivity of acyl halide compounds towards nucleophiles. AC was used in large excess due to the occurring of competitive hydrolytic reactions with water used as cosolvent in the synthetic process and the poor availability of the reacting groups of ulvan caused by the aggregation behaviour of the polysaccharide in aqueous solution.[25] The reaction was carried out at 4°C in order to prevent the undesired polymerization of acryloyl groups and hydrolysis of the formed carboxyl ester linkages. Ulvan was pretreated with concentrated NaOH solution before reaction with AC in order to increase the nucleophilicity of the hydroxyl groups of the polysaccharide toward the addition process. Two reactions between ulvan and AC, which differed for the concentration of NaOH used in the activation process, were carried out (Table 1).

In both reactions NaOH was used in large excess since the activation process of hydroxyl groups of ulvan occurring by deprotonation, was hampered by the poor acidity of alcoholic protons.

UA was successfully synthesized as evidenced by FT-IR and ^{1}HNMR spectroscopy (Figure 3).

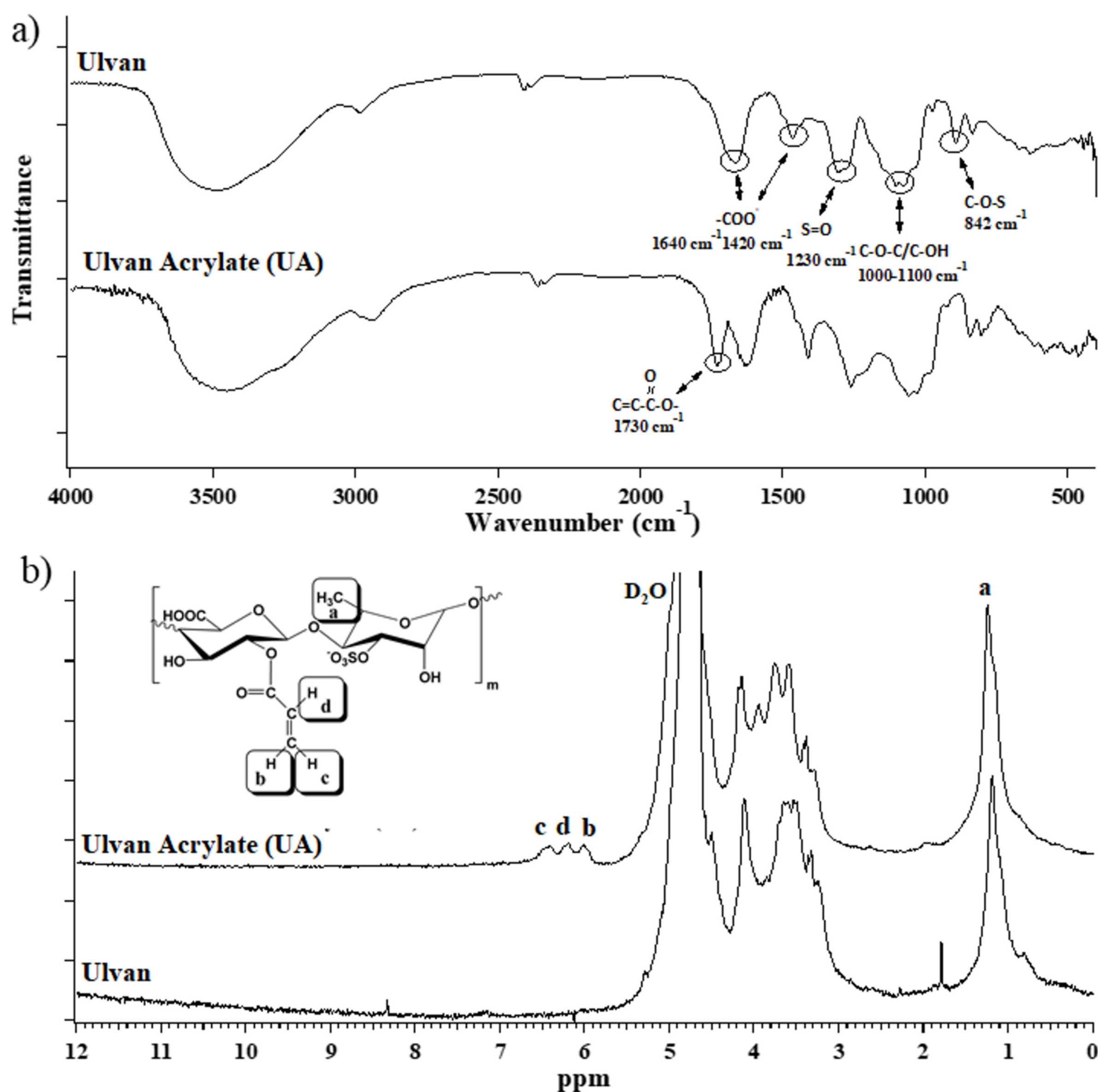

Figure 3 a) FT-IR and b) ^{1}HNMR spectra of ulvan and UA and with relevant peak attribution. FT-IR spectrum of UA revealed the presence of an absorption peak recorded at 1730 cm^{-1} not found in the spectrum of pristine ulvan which was attributed to the stretching vibration of an α,β-unsaturated ester bond (Figure 3a). The modification of ulvan with acryloyl groups was confirmed by ^{1}HNMR analysis, as evidenced by the presence of the peaks in the spectral region laying between 6.0 and 6.5 ppm which was attributed to the resonance of the alkenyl protons of UA (Figure 3b).

^{1}HNMR analysis was used to determine the substitution degree (DS) of UA, defined as the mean number of acryloyl groups introduced in each repeating unit of the polysaccharide. DS was calculated as the ratio between the normalized area of the signals of the alkenyl protons of acrylate group in UA (**b**, **c** and **d**, Figure 3b) to that relevant to the methyl protons of the rhamnose units of UA (**a**, Figure 3b). The use of larger amount of NaOH led to a significant increase of the substitution degree of ulvan thus highlighting the importance of the activation step of the hydroxyl groups of the polysaccharide in the modification process (Table 1).

3.1.2 Synthesis of (UA-PNVCL) based nanogels

UA represents a chemically versatile platform of biomaterials, which could be easily modified according to the desired applications. The pendant double bonds could copolymerize with a suitable co-monomer to obtain its grafted chains onto the polysaccharide. Our strategy was to endow UA with thermoresponsive properties by copolymerizing NVCL onto the acryloyl side chains of ulvan by using UV irradiation and IRGACURE® 2959 as a cytocompatible photoinitiator.[26] UV mediated free radical polymerization was selected as straightforward and rapid method to obtain polymeric materials by using readily accessible equipment. According to the adopted experimental conditions the grafting copolymerization of NVCL onto UA could selectively lead to the formation of branched copolymers, hydrogels or nanogels. The substitution degree and concentration of UA represented crucial parameters to selectively obtain the desired macromolecular system since UA acted as a multifunctional macromer that could function as a macro-crosslinker for the growing PNVCL polymeric chains.[27] Nanogels are polymeric networks dispersed as colloidal systems which are usually synthesized at high dilution conditions by means of crosslinking reactions which can occur either on pre-formed colloidal systems or vinylic monomers in presence of multifunctional agents by ‘‘free radical crosslinking copolymerization’’ (RCC).[27] Intermacromolecular crosslinking, which invariably leads to the formation of a polymeric network on macroscopic

scale, could be prevented either kinetically or thermodynamically by carrying out the process under diluted conditions.[28] This is commonly achieved either by using low monomer and/or crosslinker concentration and a large monomer/crosslinker ratio or stopping the polymerization at low monomer conversion. Alternative strategies to obtain nanogels rely on carrying out the polymerization/crosslinking reactions under heterogeneous conditions by promoting the processes on nanoscale area through the addition of surfactant agents.[27] Our strategy was to use UA as a novel macromer/crosslinking agent of natural origin to get rid of the synthetic crosslinkers traditionally used for the synthesis of nanogels.[28] The fabrication of thermoresponsive nanogels is usually carried out by precipitation polymerization since the process occurs above the LCST temperature of the growing thermoresponsive chain.[29]

The synthetic process designed to obtain (UA-PNVCL) based nanogels was an hybrid approach between RCC, precipitation polymerization and crosslinking reaction of pre-formed colloidal systems since ulvan macromolecules naturally organize in water as supramolecular assemblies.[25] The mild amphiphilic nature of ulvan provided by the concurrent presence of hydrophilic (hydroxyl, sulphate and carboxyl) and hydrophobic (methyl) groups allowing for the development of protocols for the preparation of surfactant free nanogels which are particularly attractive for biomedical uses. The procedure adopted to fabricate UA-PNVCL-based nanogels was purposely designed to be straightforward in order to be possibly implemented on larger scale. An aqueous mixture comprising UA, NVCL monomer and a cytocompatible photoinitiator was firstly exposed for brief times to UV irradiation and then purified by extensive dialysis against water. Different experimental parameters such as UA type, UA and NVCL concentration and time of UV exposure were investigated. Nanogels dispersions, at the end of the irradiation processes, appeared opalescent since the polymerization reactions took place above the LCST value of the growing NVCL macromolecular chains, due to the heating effect of the irradiating high pressure UV lamp (70°C). Upon cooling the UA-PNVCL-based nanogels dispersions turned transparent

evidencing the thermal transition occurring to the synthesized systems. The absence of opalescence recorded in the solution at the end of the irradiation process was considered as a valid test of unsuccessful formation of nanogel dispersions. Most of the tested conditions revealed unsuitable to provide the formation of the expected nanogels since they led either to macrogelation or to the formation of poorly thermoresponsive materials. The unsuccessful formation of nanogels in most of the adopted experimental conditions could be interpreted by taking into account that according to the reactivity ratios NVCL preferably copolymerize with acrylate units leading to crosslinking reaction and/or short grafted chains in presence of UA macromer.[30] Indeed UA-NVCL-based nanogels were successfully synthesized by using NVCL concentration value close to its solubility limit in water (4.0 w/v %) in order to obtain grafted NVCL macromolecular chains onto UA of suitable length to provide thermoresponsive behaviour in water (Table 2).

NVCL monomer conversion and weight composition of the developed nanogels were obtained by weighing method by calculating the difference between the amount of product isolated after purification and the amount of UA used in the polymerization process, assuming that all UA used in the polymerization reaction was recovered in the final product. The intended hypothesis was validated by taking into account that the purification step of the developed nanogels was carried out by using the same dialysis equipment employed for UA purification. The blank experiment, carried out in absence of UA, did not lead to the isolation of any product after purification, evidencing the inability to synthesize PNVCL homopolymer with higher molecular weight than the molecular weight cut-off (MWCO) of the adopted dialysis membrane (MWCO = 3500 Da) under the experimental conditions optimized for nanogel fabrication (Table 2). The result obtained in the blank experiment represented a valid evidence for claiming that the detection of NVCL-based material in the isolated nanogels arose from the grafting copolymerization of NVCL to UA macromer and not to the presence of a physical mixture between the unreacted UA and the NVCL homopolymer. The

experimental conditions optimized to obtain nanogels from UA-1 macromer were more critical than those adopted for UA-2 macromer since macrogelation frequently occurred due to the higher substitution degree of UA-1 with acrylate units (UA 1-2, Table 1). UA-2 led to the formation of nanogels both in the same experimental conditions optimized for UA-1 (UA-2-1, Table 2) and by using half amount of macromer as well (UA-2-2, Table 2). NVCL monomer conversion and NVCL/UA values calculated in the developed nanogels represented an underestimation of the actual reactivity of the monomer under the adopted experimental conditions since they referred only to the fraction grafted onto UA without considering homopolymerization of NVCL. The recorded low values could be explained by considering the higher reactivity of NVCL toward copolymerization instead of homopolymerization in presence of acrylate units and the use of low amount of UA to avoid macrogelation. [30] Indeed the significantly low monomer conversion of NVCL recorded in the synthesis of UA-2-2 could be attributed either to the low concentration and substitution degree of UA-2 used (Table 2). Likewise, the lower amount of NVCL incorporated into UA-1-1 nanogels synthesized in the same experimental conditions adopted for UA-2-1 could be ascribed to the larger extent of copolymerization reactions occurring between the growing PNVCL chains and UA-1 macromer (Table 2). FT-IR analysis of the obtained samples confirmed both the presence and the relative composition of PNVCL and ulvan in the developed nanogels calculated by weighing method (Figure 4).

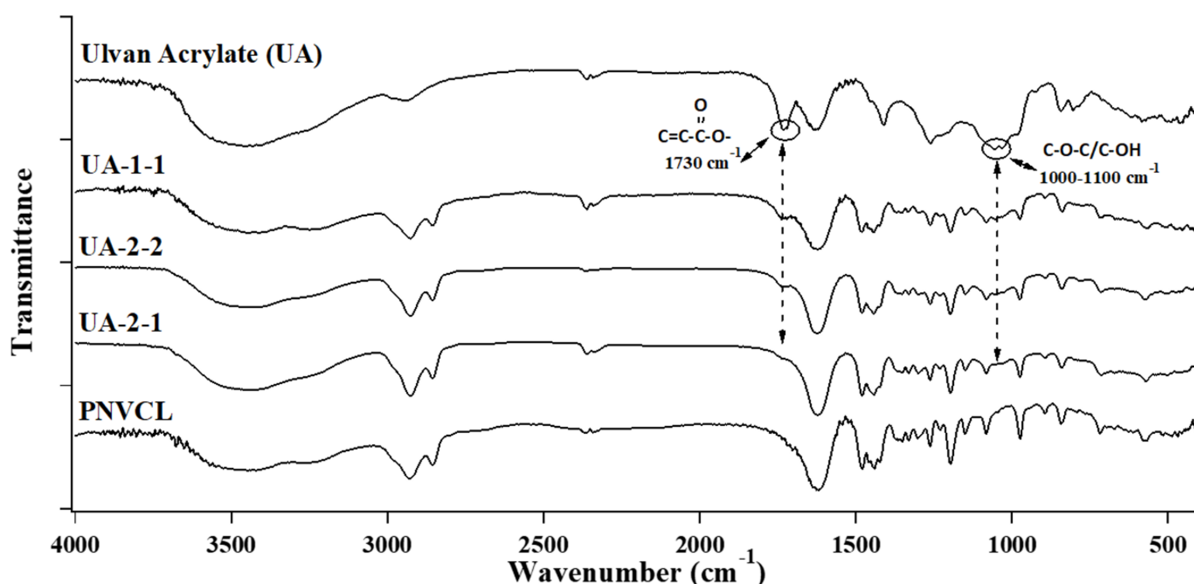

Figure 4 FT-IR spectra of the synthesized nanogels superimposed to those of UA e PNVCL used as reference.

FT-IR spectra of the synthesized materials resulted superimposable to that of PNVCL hence confirming the results obtained by weighing method which evidenced a large incorporation of PNVCL within the obtained nanogels (Figure 4), The presence of UA in the spectra of the synthesized materials emerged from two absorption bands occurring at 1730 cm^{-1} and 1100-1000 cm^{-1} which were attributed to the stretching vibration of unreacted α,β-unsaturated ester bond and new formed saturated carboxyl ester bond and carbon-oxygen single bond respectively (Figure 4). The intensity contribution of UA bands to the spectra of the analysed nanogels reflected the NVCL/UA ratios calculated by the weighing method (Figure 4, Table 2).

The synthesized nanogels were further characterized by DSC analysis in order to confirm their copolymeric nature and possibly estimate the extent of crosslinking present within their structure. Indeed both the chemical composition and the presence of crosslinked material are known to affect the glass transition temperature (Tg) of polymeric materials. Likewise a copolymer is usually discriminated from a physical mixture of the constituting homopolymers by the detection of a single Tg whose value lies in between those of the single

macromolecules. Prior to analysis the samples were conditioned at 70 °C under vacuum in order to remove unbound water which is known to negatively interfere with Tg determination due to its plasticizing effect.[13] The thermograms obtained by the second heating cycle of the synthesized samples evidenced the presence of a single Tg value comprised in between those of UA and PNVCL and hence confirming the copolymeric nature of the developed materials (Figure 5a).

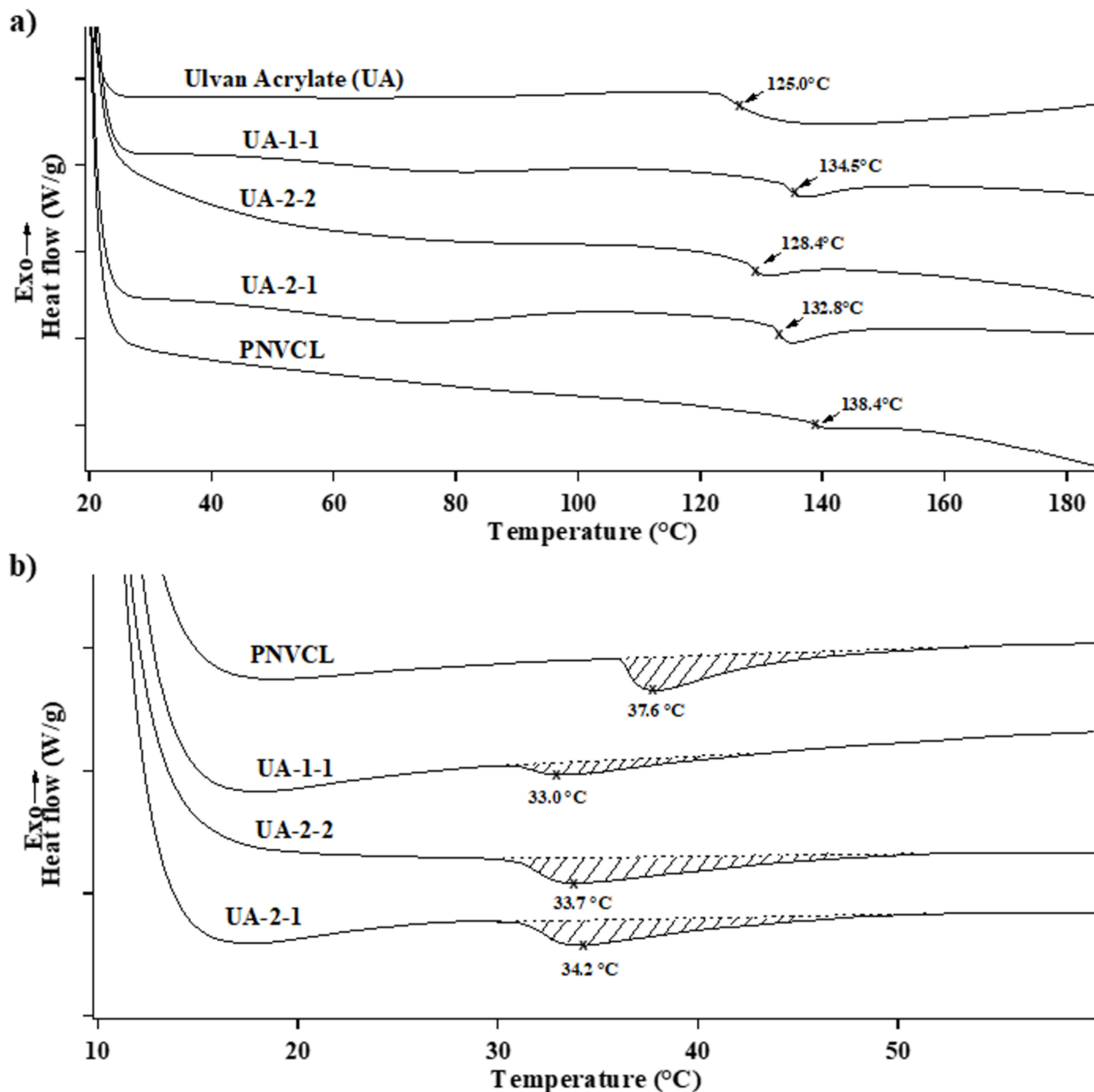

Figure 5 a) DSC thermograms with corresponding Tg values of the synthesized nanogels superimposed to those of UA e PNVCL used as reference and b) DSC thermograms of the synthesized nanogel dispersions in PBS (0.01M, pH 7.4) with corresponding VPTT values superimposed to that of PNVCL used as reference.

Tg values of UA-2-1 and UA-2-2 were consistent with the NVCL/UA ratios obtained by weighing analysis indicating a negligible contribution from crosslinking reactions. Glass transition of UA-1-1 occurred at higher temperature than the value expected from NVCL/UA composition due to a major contribution provided by the presence of crosslinked material.

Collectively the synthesized UA-1-1, UA-2-1, UA-2-2 were firstly selected as thermoresponsive materials by visual observation. FT-IR and DSC analysis confirmed the results obtained by the weighing method evidencing the formation of grafted copolymers, which resulted significantly crosslinked by using UA-1 as macromer. The presence of crosslinking in the systems UA-2-1 and UA-2-2 did not emerge clearly from DSC analysis and thus were submitted to further characterization.

3.2 Determination of thermal responsivity of poly (N-vinylcaprolactam)-g-Ulvan (UA-PNVCL) based nanogels

The thermal responsiveness of the synthesized UA-PNVCL based nanogels was evidenced by the reversible change from transparent to opalescent liquid that occurred during the polymerization process of the relevant dispersion. However for being considered for drug delivery applications the corresponding phase transitions of nanogels should meet stringent requirements comprising a sharp volume change occurring in the range of physiological temperature. To this aim the synthesized materials were submitted either to thermal analysis (DSC) and dynamic light scattering analysis (DLS) in order to evaluate their feasibility for being used as thermoresponsive nanocarriers in biomedical applications.

3.2.1 Thermal analysis

The thermal sensitivity of the synthesized nanogels was evaluated by DSC analysis since it allowed for a rapid determination of the heat associated to the phase transition from hydrophilic to hydrophobic of the thermoresponsive component of the nanogels. This method, commonly applied to determine the lower critical solution temperature (LCST) of

thermoreversible hydrogels, could be easily implemented to thermoresponsive nanogels for the detection of the 'volume phase transition temperature' (VPTT), which is generally close to the LCST of the corresponding homopolymer.[23,27,31-32] Indeed the structural rearrangement occurring during the phase transition from liquid to gel in the solutions of physically crosslinked thermoresponsive polymers, usually provokes a volume variation in thermoresponsive nanogel particles.[27] Nanogel solutions were analysed by using PBS (0.01 M, pH 7.4) as solvent in order to determine the VPTT values under simulated physiological conditions (Figure 5b).

The VPTT values which were determined as the minimum of the endothermic peaks of the recorded thermograms revealed consistent with the NVCL/UA ratios of the nanogels calculated by the weighing method (Figure 5b, Table 2). The DSC curve profiles of the analysed nanogels approached that of PNVCL thermogram, used as reference, as much as the amount of NVCL in the tested samples increased. However the VPTT values of synthesized nanogels were fairly lower than the LCST value of PNVCL hence evidencing the favouring activity of UA in inducing the phase transition of the grafted PNVCL chains even at high NVCL/UA ratios. Indeed, the VPTT of all the analysed nanogels occurred below the physiological temperature highlighting the thermodynamic suitability of the developed nanogels for being applied in biomedical fields.

3.2.2 Dimensional analysis

The fabricated nanogels were investigated by Dynamic Light Scattering (DLS) in order to detect their dimensional responsiveness in the range of VPTT values recorded by DSC analysis. DLS is usually applied for the determination of the dimensional distribution of colloidal systems and constitutes the most representative method for detecting the thermoresponsive behaviour of nanogels by measuring their size variability with temperature. The evolution of the mean hydrodynamic diameter of the analysed samples recorded in the

range of physiological temperature was consistent with those of other PNVCL-based nanogels described by the literature (Figure 6)[13-14]

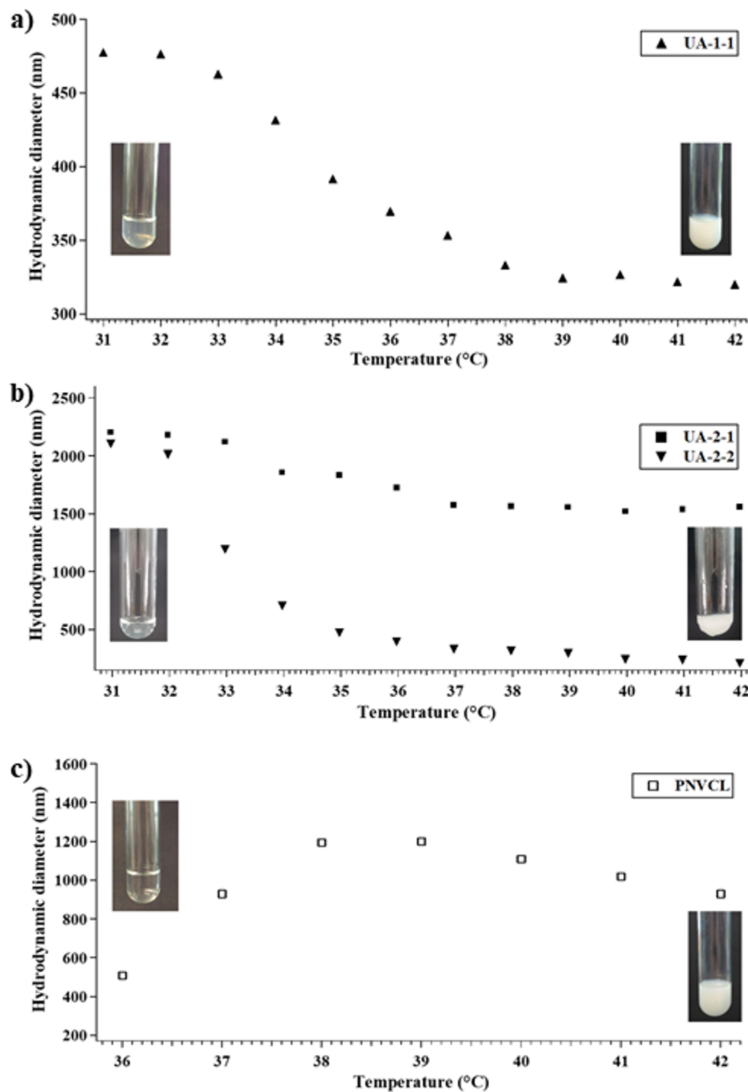

Figure 6 DLS analysis of a) UA-1-1, b) UA-2-1, UA-2-2, c) PNVCL samples carried out in the range of physiological temperature. The pictures of the dispersions taken at room temperature and 42°C were reported within each corresponding DLS curve.

The DLS curve of PNVCL did not show a regular variation of the hydrodynamic diameter with temperature due to the absence of crosslinking hence highlighting the activity of UA as crosslinker in the synthesized nanogels.

Upon exposure to increasing temperature all the synthesized materials experienced dimensional shrinkage especially in the range close to the VPTT values recorded by DSC analysis. This behaviour was triggered by the phase transition from hydrophilic to

hydrophobic occurring to the PNVCL macromolecular chains grafted onto the UA macromer which ultimately led to a volume contraction of the nanogels due to the elimination of molecules of water.[33] The dimensional shrinking occurred to the analysed samples in the range of physiological temperature was confirmed by visual observation of the relevant dispersions whose aspect turned from weakly turbid at room temperature to opalescent at 42°C (Figure 6). The hydrodynamic diameter values of the synthesized materials recorded at 31°C which ranged from nanoscale (UA-1-1) to microscale dimensions (UA-2-1, UA-2-2) were related to the type of UA used and hence the substitution degree of the materials (Figure 6). The larger hydrodynamic diameters of UA-2-1 and UA-2-2 were likely triggered by the lower density of acrylate units present in UA-2 macromer, which ultimately led to the grafting copolymerization of longer PNVCL pendant chains onto their initiating groups (Table 1 and Table 2). The hydrodynamic diameter value of UA-1-1 at 31°C was similar to that of ulvan macromolecules which naturally assemble in water as nanosized aggregates thus suggesting the presence of chains with short NVCL sequences grafted onto UA-1-1.[25] The difference of dimensions experienced by the nanogels during the phase transition was not found to depend univocally with the amount and the substitution degree of UA used to fabricate the materials (Table 3).[16]

Table 3. Hydrodynamic diameter variation experienced by the synthesized nanogels in the range of temperature (31°C-42°C) measured by DLS analysis

Sample	**NVCL/UA[a] (w/w)**	**ΔHD[b] (nm)**
UA-1-1	7.1	150
UA-2-1	11.8	700
UA-2-2	8.9	1800

a: Composition of the synthesized nanogel calculated by means of weighing method.
b: ΔHD = Mean hydrodynamic diameter (42°C) – Mean hydrodynamic diameter (31°C).

Likewise ΔHD was correlated to mean length and number of the PNVCL chains involved in the intermolecular crosslinking acting as macromolecular springs during the phase transitions. The size variation of UA-2-1 nanogels occurred less sharply than in the other analysed samples indicating that most of the grafted PNVCL chains were not involved in crosslinking reactions although DSC analysis did not prove a significant crosslinking in UA-2-2 (Figure 6). However ΔHD values calculated for UA-2-2 suggested that longer PNVCL chains than those involved in UA-1-1 were participating to intermacromolecular crosslinking hence improving the degree of flexibility of the material and decreasing the Tg value by plasticizing effect. The mean hydrodynamic diameters of the synthesized nanogels at 42 °C fairly accounted for the NVCL/UA ratios obtained by weighing analysis since the extended PNVCL chains collapsed into a dense nanosized aggregate whose dimension was less dependent on the length of the grafted macromolecular chains (Figure 6).

Collectively DLS analysis evidenced the suitability of the fabricated nanogels to physically respond to thermal stimuli in the range of physiological temperature hence resulting valid candidates for the controlled release of drugs.

3.3 Protein encapsulation on (UA-PNVCL) based nanogels

BSA was selected as a model compound to investigate the feasibility of the developed thermoresponsive ulvan-based nanogels for being used as protein encapsulating carriers. Among the synthesized matrices UA-1-1 and UA-2-2 were considered as the most promising candidates since they more rapidly underwent to volume changes in response to thermal stimuli (Figure 6). BSA was loaded into the selected nanogels through adsorption by means of the incubation method since it represented a more straightforward and mild technique for protein loading into polymeric carriers although less efficient than encapsulation method.[34-35] Protein solutions containing the nanogels dispersed in PBS (0.01 M, pH 7.4) were allowed to stand at 4°C for 24 hours in order to favour their adsorption into the core of the nanosized

materials since it convert into the extended conformation at the selected temperature.[34] BSA loading was indirectly determined by measuring the concentration of the unloaded protein present in the supernatant liquid of the centrifuged dispersions at the end of the encapsulation process (**Equation 2.1**). Quantification was carried out by colorimetric method by means of using the BCA assay and BSA as standard for the calibration curve recorded in the concentration range 1-200 µg/mL. Three blanks were separately prepared in order to assess either the contribute of each nanogel dispersion to the colorimetric assay and the maximum absorbance of unloaded protein whose value was needed for protein loading and loading efficiency calculation (Table 4).

Table 4. Loading efficiency of BSA onto UA-PNVCL nanogels determined by BCA assay.

Sample	**NVCL/UA[a] (wt/wt)**	**Nanogel (mg)**	**BSA (mg)**	**A_{565}[b]**	**BSA[c] (mg)**	**Protein Loading[d] (mg)**	**Loading Efficiency[e] (%)**
UA-1-1 blank	7.1	10	-	2.12	-	-	-
UA-1-1	7.1	10	0.15	2.99	0.091	0.044	34.0
UA-2-2 blank	8.9	10	-	1.83	-	-	-
UA-2-2	8.9	10	0.15	2.88	0.113	0.022	17.0
BSA blank	-	-	0.15	1.23	0.135		

a: Composition of the synthesized nanogel calculated by means of weighing method.
b: Absorbance at 565 nm of the supernatant liquids separated by centrifugation by the corresponding nanogel dispersion.
c: amount of unloaded protein found in the supernatant liquids calculated by using the BCA assay
d: calculated according to equation 2.1 by using BSA blank as total protein value
e: calculated according to equation 2.2 by using BSA blank as total protein value

The loading efficiencies calculated for both the analysed samples were slightly lower than the values reported in literature obtained by using different nanogel devices due to electronic repulsion occurring between ulvan and BSA which are negatively charged at pH 7.4.[34] However the loading efficiency of UA-1-1 was significantly higher than that of UA-2-2

maybe due to the increased surface exposed to protein adsorption by UA-1-1 nanogels than UA-2-2 microgels indicating the importance of the dimensions of the colloidal carriers on the efficiency of the encapsulation processes (Figure 6).

4. Conclusions

The present study aimed at promoting the valorization of waste algal biomasses, typically involved in environmental concerning issues, into a resource of high-value added polymers for biomedical applications. In particular ulvan, an underexploited sulphated polysaccharide of algal origins displaying unique physical-chemical and biological properties, was successfully employed for the fabrication of novel thermoresponsive nanogels for the controlled release of bioactive compounds. The innovation of the present study was brought by the unprecedented use of ulvan in the synthesis of thermoresponsive nanogels which was further strengthened by the novel application of a natural crosslinker instead of the synthetic crosslinker agents conventionally used in this kind of applications.

Nanogels were synthesized through a rapid and facile procedure by radical copolymerization of NVCL onto acrylate modified ulvan macromer mediated by UV irradiation. In the optimized experimental conditions ulvan-acrylate acted both as a grafting-from macromer and crosslinking agent for PNVCL chains to link onto its lateral groups. The mild amphiphilic character of ulvan provided by the concurrent presence of hydrophilic and hydrophobic groups within its polymeric structure, allowed for the development of colloidally stable nanogel dispersions without the need of surfactants. The most promising nanogels were submitted to preliminary investigations for their ability to encapsulate proteins, which represent an advantageous class of therapeutic agents recently used for the treatment of severe diseases. A slight increase of the amount of ulvan incorporated in the developed nanogels was found to significantly improve the performances the synthesized materials by increasing the loading efficiency of protein encapsulation and the thermoresponsive behavior. Collectively

the use of ulvan could represented a significant progress in the development of nanogels for biomedical applications since it allows avoiding synthetic crosslinkers and surfactant agents conventionally used to synthesize these materials. Moreover the characteristics of the obtained devices could be easily tuned through a slight variation of the amount and/or substitution degree of the ulvan used. Finally, the nanogels developed in this study will be further investigated as injectable systems for the *in situ* release of therapeutic proteins (e.g. growth factors, cytokines and monoclonal antibodies), which usually require severe administration by long-term infusions due to their *in vivo* short half-life and physicochemical instability

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Table of Contents

Ulvan was employed to develop a novel macromolecular crosslinker of natural origin for the preparation of thermoresponsive nanogels for biomedical applications.

Andrea Morelli, Dario Puppi, Victoria Cheptene, Dunia Disgraziati, Giacomo Ruggeri, Federica Chiellini*

Design, preparation and characterization of thermoresponsive hybrid nanogels using a novel ulvan-acrylate crosslinker as potential carriers for protein encapsulation.