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Ulvan: A Versatile Platform of Biomaterials from Renewable Resources

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1. Introduction

Biomass represents an abundant renewable resource for the production of bioenergy and biomaterials and its exploitation could lead to overcome the dependence from petroleum resources. Indeed fossil energy and chemical sources are not unlimited and there is a critical need to turn the current way of life back to a sustainable manner. The conversion of biomasses into high value chemicals, energy and materials is nowadays gaining more and more attention and represents the final goal of the "Industrial Biorefinering". Indeed Biorefinery aims at the optimum exploitation of biomass resources for the production of materials that eventually might replace the conventional products from fossil/non renewable resources, thus decisively contributing to the development of a sustainable system. The great challenge in which Biorefinering is involved is the possibility of creating high value products from low value biomasses. In this view, the feasibility of using starting materials obtainable from organic waste sources (agricultural, municipal and industrial waste) or having harmful effects on the environment (algae) as feedstock can represent the strategy of election for the production of sustainable materials.

To this aim algae could represent a potentially advantageous biomass to be explored since they are very abundant and cheap and very often involved in uncontrolled proliferation processes detrimental for marine and aquatic environments (Barghini et al., 2010, Chiellini et al., 2008, 2009, Fletcher, 1996). Today most of the naturally produced and harvested algal biomass is an unused resource and often is left to decompose on the shore creating waste problems (Morand et al., 2006). The current use of this huge underexploited biomass is mainly limited to food consumption and as bio-fertilizer, but its potentiality as renewable and sustainable feedstock for energy and material production is gaining more and more attention (Demirbas A. & Demirbas M.F., 2011). Indeed microalgae have been considered to be an excellent source for biodiesel production since are characterized by high growth rates and high population densities, ideal for intensive agriculture and may contain huge lipid amounts, needed for fuel production (Christi, 2007). Besides biodiesel, algae can be cultivated and can be used as a feedstock for the production of bioethanol (John et al., 2011). In particular macroalgae (seaweed) can produce huge amount of carbohydrates per year

(Matsumoto et al., 2003) that suitably processed through specific fermentation processes would provide renewable and sustainable biofuel.

Algae represent also an advantageous resource of chemicals and building block materials that can be tailored through proper biorefinering processes according to the different envisaged applications. The rising demand for natural instead of synthetic materials especially in biomedical applications where high biocompatibility and no adverse effects for the host organism are required (Mano et al., 2007), has led to an outburst of scientific papers involved in the study of biobased materials. Among these, polysaccharides could represent the best candidate since abundant, biocompatible and displaying a pronounced chemical versatility given by the great number of chemical functionalities present in their structures. The list of known natural carbohydrates is continuously growing, owing to new discoveries in animal and plant material (Tsai, 2007). They can be used in their native form or after proper chemical modifications made according to the final applications (d'Ayala et al., 2008). The use of polysaccharides of animal origin (e.g. heparin and hyaluronic acid) in biomedical applications is not straightforward since it can raise concerns about immunogenicity and risk of disease transmission (Stevens, 2008) Indeed these materials require very accurate purification treatments aimed to free them from biological contaminants, in contrary to polysaccharides of plant (e.g. cellulose and starch) or algal origin (e.g. alginate). Polysaccharides of algal origins are gaining particular attention due to their abundance, renewability (Matsumoto et al., 2003) and to their peculiar chemical composition not found in any other organisms. Over the last few years medical and pharmaceutical industries have shown an increasing interest in alginate (d'Ayala et al., 2008), an anionic polysaccharide widely distributed in the cell walls of brown algae. This biopolymer has been largely used for its gel forming properties. Due to its non-toxicity, unique tissue compatibility, and biodegradability, alginate has been studied extensively in tissue engineering, including the regeneration of skin (Hashimoto et al., 2004), cartilage (Bouhadir et al., 2001), bone (Alsberg et al., 2001), liver (Chung et al., 2002) and cardiac tissue (Dar et al., 2002).

A very intriguing feature that distinguishes algal biomass from other resources is that it contains large amounts of sulphated polysaccharides, whose beneficial biological properties (Wijesekara et al., 2011) prompt scientists to increase their use in the biomedical fields. Indeed the presence and the distribution of sulphate groups in these polysaccharides are reported to play an important role in the antiviral (Damonte et al., 2004), anticoagulant (Melo et al., 2004), antioxidant (Rocha de Souza et al., 2007) and anticancer (Athukorala et al., 2009) activity of these materials.

The chemical composition of the sulphated polysaccharides extracted from algae, including the degree and the distribution of the sulphate groups, varies according to the species, and the ecophysiological origin of the algal sources (Rioux et al., 2007). Anyhow, a structural differentiation depending on the different taxonomic classification of the algal origin, has been found. According to the mentioned classification the major sulphated polysaccharides found in marine algae include fucoidan from brown algae, carrageenan from red algae and ulvan obtained from green algae (Figure 1).

Ulvan polysaccharides possess unique structural properties since the repeating unit shares chemical affinity with glycoaminoglycan such as hyaluronan and chondroitin sulphate due to its content of glucuronic acid and sulphate (Figure 2).

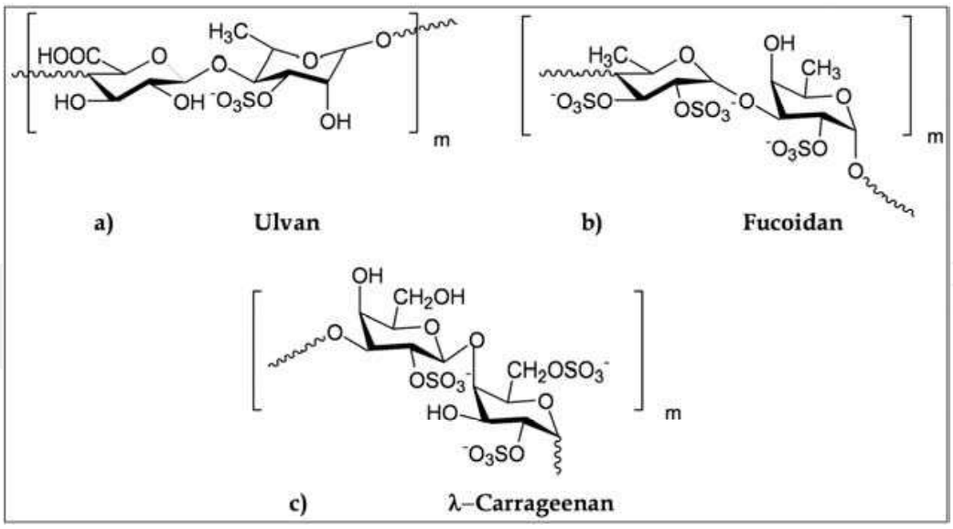

Fig. 1. Chemical structure of the dimeric repeating unit of a) Ulvan, b) Fucoidan, c) λ - Carrageenan.

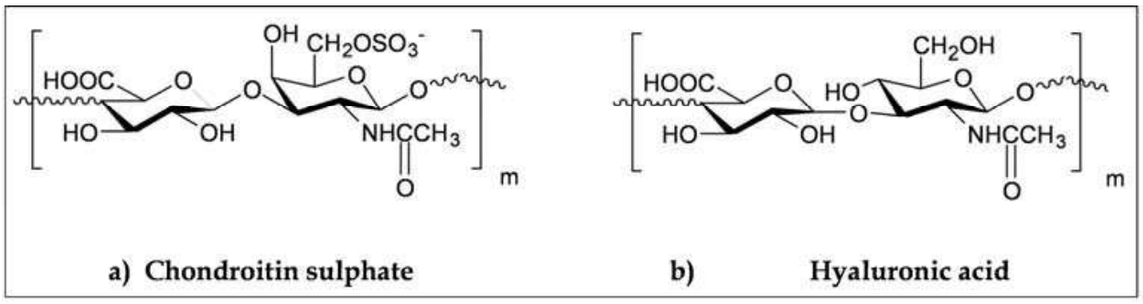

Fig. 2. Chemical structure of the dimeric repeating unit of a) Chondroitin sulphate, b) Hyaluronic acid.

This resemblance and the possibility of obtaining this material from cheap and renewable resources make it worthwhile a deeper investigation on the biological activity and the feasibility of using this polysaccharide for biomedical applications.

2. Ulvan properties

Ulvales (Chlorophyta) are very common seaweeds distributed worldwide. The two main *genera Ulva* and *Enteromorpha* are sadly known for being involved in processes detrimental for the aquatic environment. Indeed this algal biomass proliferates very quickly in eutrophic coastal and lagoon waters in the form of "green tides" leading up to hypoxia and death of most of aquatic organisms (Morand & Brian, 1996). Environmental concerns arise also for the disposal of this huge biomass that is mostly left do degrade on the shore creating nuisance problems, so that its exploitation could represent a remedy to related environmental and economical concerns.

To date, this biomass has very low added value and its use is limited to food consumption (Bobin-Dudigeon et al., 1997) composting (Mazè et al., 1993) and methane production

(Brand & Morand, 1997) but as it will be stressed in the following part of the chapter, the chemicals and polymers of this underexploited biomass along with their abundance, biological properties and "renewability" represent a potential source to be explored.

2.1 Chemical-physical properties

2.1.1 Ulvan composition

Green algae such as *Ulva sp.* are known to contain high amounts of good-quality protein, carbohydrate, vitamins and minerals (Taboada et al., 2010). Among these, polysaccharides are gaining increasing attention as they possess unique physical and chemical properties representing a versatile material platform for potential biological applications.

Ulvan represents a class of sulphated heteropolysaccharide extracted from the cell wall of green seaweeds belonging to *Ulva sp.* whose composition has been extensively debated (Lahaye & Robic, 2007; Robic et al., 2009;) and showed to vary according to several factors including the period of collection, the ecophysiological growth conditions, the taxonomic origins and the post-collection treatment of the algal sources (Lahaye & Robic, 2007).

Four types of polysaccharides are reported to be contained in the biomass of *Ulva sp.*, including the water soluble Ulvan and insoluble cellulose as major one and an alkali-soluble linear xyloglucan and glucuronan in minor amounts (Lahaye & Robic, 2007). Ulvan represents the major biopolymeric fraction of the cell wall having the function of maintaining the osmolar stability and protection of the cell (Paradossi et al., 2002). As usually found in polysaccharides present into the cell walls, Ulvan is present in close association with proteins and the conventional methods of extraction and purification resulted not completely effective in the removal of the protein fraction even after a specific deproteinization protocol (Alves et al., 2010).

Extraction is conventionally achieved by using warm water solution (80-90°C) containing ammonium oxalate as divalent cation chelator and the recovery of Ulvan is generally obtained by precipitation in ethanol. The yield of extraction usually ranges from 8% to 29% of the algal dry weight depending on the applied purification procedure (Lahaye & Axelos, 1993; Lahaye et al., 1994).

The sugar composition of Ulvan is extremely variable but rhamnose, xylose, glucuronic and iduronic acid and the presence of sulphate groups have been identified as the main constituents of the polymer (Paradossi et al., 2002; Robic et al., 2009). These monomers are arranged in an essentially linear fashion even though a slight degree of branching has been found (Lahaye & Robic, 2007). The chemical heterogeneity of Ulvan is partially striken by a "structural motif" found within the heteropolymer chain essentially given by the presence of repeating dimeric sequences constituted by aldobiuronic acid disaccharides designated as type A (glucurorhamnose 3-sulphate, A_{3s}) and type B (iduronorhamnose 3-sulphate, B_{3s}) (Figure 3).

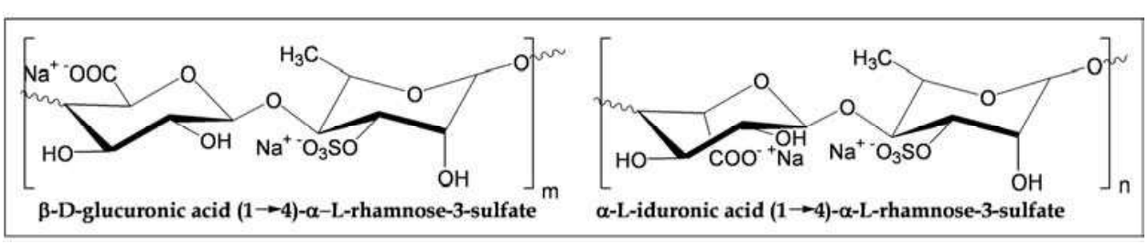

Fig. 3 Structure of the main disaccharide repeating units in Ulvan.

The most striking feature that distinguishes the chemical composition of Ulvan from that of the other polysaccharides of marine origin is, therefore, the presence of uncommon sugar such as iduronic and sulphated rhamnose displaying a close similarity with mammalian glycosaminoglycans. To this view Ulvan and related polysaccharides could represent an abundant and cheap feedstock for the substitution of heparinoid substances commonly used in biomedical applications solving the problems related to their isolation and purification (Alban et al., 2002).

2.1.2 Ulvan conformation

The physical properties of polymeric materials are deeply affected by the association and conformation assumed by the constituting chains in the final product. The balance between ordered crystalline and disordered amorphous structures dictates the ultimate mechanical properties of the polymeric material. Indeed the possibility of forming crystalline regions inside a polymeric structure could even generate physical crosslinks between the chains inducing ultimately to the formation of stiff networks, as in the case of polyvinyl alcohol (Ricciardi et al, 2005). The achievement of suitable mechanical properties for a material to be used in biomedical applications, namely tissue engineering, represent a key requirement to fulfil since the final product must provide a physical support for the cell growth and differentiation.

Past investigations on this issue revealed an essentially disordered conformation of Ulvan (Paradossi et al., 1999) mainly induced by the heterogeneous chemical composition of this polysaccharide. The local regularity given by the repeating aldobiuronic units, denominated as A_{3s} and B_{3s} (Figure 3), is believed to be sufficient for the formation of transient "junction-zones" responsible for the formation of the weak gel that ulvan is known to perform in nature (Paradossi et al., 2002). The stability of these ordered structures can be affected by the attractive and repulsive interactions that form between the functional groups of the polysaccharide, and in particular by the electrostatic forces. Ulvan is an anionic polyelectrolyte as it contains carboxylic and sulphate groups inside its structure, so that its net charge strongly depends on the pH and ionic strength of the working medium. The net charge on Ulvan is found to affect the conformation of its polymeric chains and ultimately controls the order to disorder transitions given by the locally regular sequences (Paradossi et al., 2002). The conformational change from an ordered structure present in the uncharged chain, i.e. the protonated form of ulvan, toward a disordered state, happens when a critical charge density is reached and is induced only in the chemically regular portions of the chains. The structures of the ordered sequences have been hypothesized on the basis of molecular modelling calculations and are compatible with the formation of helical conformations inside homogeneous portions of the chains containing the repeating units A_{3s} and B_{3s} (Paradossi et al., 2002).

The presence of ordered structures limited only in the regular sequences of the Ulvan polymeric chains is not sufficient to provide enough "junction-zones" for the preparation of a material with mechanical properties suitable for biomedical applications. For this purpose Ulvan has to be modified through the introduction of chemical groups or molecules that increase the number of "junction-zones".

2.1.3 Ulvan morphology and solubility

The possibility of chemically modifying Ulvan is strongly dependent on the physical availability of its functional groups so that its solubility and morphology in the working

medium could affect deeply its reactivity. Ulvan has been shown to dissolve only in water due to its charged and highly hydrophilic nature. Nevertheless, the obtained solutions are not transparent, indicating the formation of microaggregates of polymeric material not fully dispersed in the solvent. Indeed TEM analysis of Ulvan revealed the presence of aggregates of spherical shaped forms partially linked by strands-like filaments (Robic et al., 2009). This necklace-like ultrastructure is usually formed by polyelectrolyte material in poor solvent conditions (Dobrynin, 2008) so that even water can not be considered a good solvent for Ulvan. The large presence of methyl groups provided by the rhamnose repeating unit has been considered responsible for the unusual hydrophobic behavior of this highly charged polysaccharide (Robic et al., 2009).

The unusual low intrinsic viscosity of Ulvan in solution can also be ascribed to the presence of condensed spherical shaped aggregates not typical for polyelectrolytes whose conformation usually expands in the form of charged filaments and leads to an increase in the viscosity (Dobrynin et al., 1995). The formation of microaggregates in solution does not allow also a reliable mass analysis of Ulvan, whose different type of aggregation affects deeply the peak distributions usually found on the GPC chromatograms (Robic et al., 2009).

Being a polyelectrolyte, both the ionic strength and the pH of the dissolving medium would affect the solubility and the morphology of Ulvan. Indeed the association of the bead-like aggregates in a necklace-type ultrastructure is promoted by the ionic interactions of carboxylated groups as demonstrated by its rupture at pH below the pKa of glucuronic acid (3.28) (Robic et al. 2009). In basic conditions (pH 13) the bead-like structures resulted to collapse into a dense homogeneous network likely prompted by the ionic interactions of carboxylate and sulphate groups. The type and amount of counter-ion in solution could also contribute to chain expansion or condensation as demonstrated by the aggregative propensity of Ulvan at low NaCl concentration observed by light scattering and rheological measurements (Lahaye & Robic, 2007).

The tendency of Ulvan to form aggregates in aqueous solution and its insolubility in almost every organic solvents limit the number of functional groups available for chemical modifications thus hampering its potential versatility. But its great number of reactive groups still present on the "free" surface exposed outside the aggregate and the possibility to optimize the solvent variables (pH and ionic strength) that affect the dispersion of the polymer in solution make Ulvan a suitable reactive platform, tailorable according to the envisaged application.

2.2 Biological activity

The possibility of using bio-based materials in almost every technological field and particularly in biomedical applications is challenging and can be considered the strategy of election for limiting environmental concerns and create a virtuous circle of sustainability.

Biomaterials possess the essential prerequisite of renewability and biocompatibility and as such are worth of deep investigations as main candidates for the substitution of synthetic petroleum-based materials, well known for being not renewable and often not biocompatible.

Biodegradability represents also an important property possessed by biomaterials and it is especially required in materials used for biomedical applications with specific reference to tissue engineering and regenerative medicine. Not only the material has to be safe but also the products of degradation should be non-toxic and easily cleared from the body. Biomaterials that other than being renewable, biocompatible and biodegradable are able to

induce a beneficial biological activity on the host organism or on the environment can be considered even more intriguing. This may be the case of Ulvan. Most of the positive health effects induced by this polysaccharide are generated by the presence of sulphate groups in its structure (Wijesekara et al., 2011). A wide list of beneficial biological effects reported by the literature span from antioxidant (Qi et al., 2006) to anticoagulant (Zhang et al., 2008), antitumor (Kaeffer et al., 1998) antihyperlipidemic (Yu et al., 2003) and immunomodulating (Leiro et al., 2007) activities, proved both *in vitro* and *in vivo*.

A brief discussion about the chemical mechanisms that trigger this bioactivity can be worth of mentioning in order to have a deeper insight on the potentiality of using this biomaterial in biomedical applications, and possibly find the "keys" to improve its biological activity.

2.2.1 Antioxidant activity

The research of new antioxidant from renewable natural resources able to scavenge free radicals can represent a virtuous strategy for preventing ROS-induced diseases.

In recent years, several classes of sulphated polysaccharides have been demonstrated to show antioxidant activity. Among them Ulvan extracted from *Ulva pertusa* is reported to play an important role as free radical scavenger *in vitro* and displayed antioxidant activity for the prevention of oxidative damage in living organisms (Qi et al., 2005). As found with other sulphated polysaccharides (Wijesekara et al., 2011) the antioxidant activity is deeply affected by the amount and distribution of sulphate groups inside the Ulvan structure.

The possibility to increase the antioxidant activity of Ulvan can be useful according to the envisaged application and has been successfully investigated both by increasing the degree of sulphation through a sulphur trioxide/N,N-dimethylformamide treatment (Qi et al., 2005) and by introducing suitable groups (acetyl and benzoyl) that can boost the activity of the native polysaccharide (Qi et al., 2006).

2.2.2 Anticoagulant activity

Heparin, a glycosaminoglycan of animal origin containing carboxylic acid and sulphate groups, has been identified and used for more than fifty years as a commercial anticoagulant and it is widely used for the prevention of venous thromboembolic disorders (Pereira et al., 2002). The heparinoid-like structure of Ulvan makes it also able to provide anticoagulant activity. Indeed this class of polysaccharides displayed the inhibition of both the intrinsic pathways of coagulation or thrombin activity and the conversion of fibrinogen to fibrin (Zhang et al., 2008). The molecular weight of the polysaccharide showed an important effect on the anticoagulant activity indicating that longer chains were necessary to achieve thrombin inhibition.

This behavior has been found to be typical of sulphated polysaccharides of marine origins whose anticoagulant activity has been correlated to the content and position of the sulphate groups inside the polymer chains (Melo et al., 2004).

The importance of finding sources of anticoagulants alternative to heparin has been arising due to the associated harmful side effects and the complex steps of purification required to face the immunological concerns and disease transmission associated with its use (Stevens, 2008). Thus the increasing demand for a safer anticoagulant therapy could be potentially

satisfied by sulphated polysaccharides obtained from abundant and safer origin and Ulvan can represent the ideal material for such purpose.

2.2.3 Immunomodulating activity

Some of the polysaccharides obtained from the cell walls of seaweeds appear to exert immunomodulatory activities in mammals as they modify the activity of macrophages. Most classes of carrageenans, sulphated polysaccharide obtained from red algae (Figure 1) are known to induce potent macrophages activation (Nacife et al., 2004.).

The structure of the repeating unit mostly found in Ulvan, resembles that typical of glycosaminoglycan, such as hyaluronic acid and chondroitin sulphate because they all contain glucuronic acid and sulphate. Chondroitin sulphate based proteoglycans are known to be produced by macrophages and human monocytes at inflammatory sites (Uhlin-Hansen et al., 1993) and the structure similarity shared with most of sulphated polysaccharides of algal origin and in particular with Ulvan could represent the trigger of the immunomodulating activity of these materials. Indeed Ulvan from *Ulva rigida* has been reported to modulate the activity of murine macrophages and the presence of sulphate groups has demonstrated to be necessary (Leiro et al., 2007). On the other side, a deeper insight into the structure-immunomodulating effect relationship of these polysaccharides would provide the possibility to obtain an anti-inflammatory effect simply by properly modifying their chemical structure.

2.2.4 Antihyperlipidemic activity

Ulvan is known to resist degradation by human endogeneous enzymes thus belonging to the dietary fibers of "sea lattuce" (Taboada et al., 2010). Dietary fibers are considered to be helpful in the prevention of pathologies related to intestinal transit dysfunctions because they act as bulking agents due to their impressive water retention capacity (Bobin-Dubigeon et al., 1997). Dietary fibers are also associated with their ability to lower cholesterol levels (Brown et al., 1999) and the presence of ion charged groups along their structure has shown to improve this beneficial activity (Guillon & Champ, 2000). The ionic groups are thought to complex with bile acids and consequently increase fecal bile acid excretion thus promoting blood cholesterol attenuation (Yu et al., 2003). Ulvan is reported to interact and binding effectively with bile acids due to its high content of negatively charged groups (Lahaye, 1991) thus potentially contributing to the antihyperlipidemic action. Indeed Ulvan has been demonstrated to effectively reduce the level of total and LDL-cholesterol concentrations in the serum and induce an increase in the daily bile excretion in rats (Yu et al., 2003). This activity has been shown to be strongly dependent on the molecular weight of the polysaccharide because a decrease in the viscosity of these materials affected negatively the interaction with bile acids.

3. Potential use of ulvan in biomedical applications

A material suitable for biomedical applications, namely tissue engineering, regenerative medicine and drug delivery, is required to be biocompatible and biodegradable and its products of degradation must be safe and easily cleared from the host organisms. Most of the materials obtained from natural resources are able to fulfil these strict requirements but

particular attention are gaining biopolymers of plant and algal origins due to their abundance and minor concerns for purification (Stevens, 2008).

Ulvan could represent an advantageous versatile platform of "unique" sulphated polysaccharides that along with their abundance and renewability would potentially display the properties that match the criteria for biomedical applications. Despite the promising properties related to this material, the use of Ulvan in the biomedical fields is not yet reported and its potentiality still remain unveiled.

A base requirement for a material suitable for tissue engineering, regenerative medicine and/or drug delivery is its insolubility in the physiological fluids not possible with most classes of polysaccharides, whose high hydrophilicity make them very akin to water molecules. Indeed materials used for the regeneration of organs or tissues must avoid dissolution in contact with body fluids thus functioning as chemically and mechanically stable scaffolds during the growth and differentiation of the implanted cells. Also polymeric materials used for drug delivery must preserve their integrity or degrade slowly in order to maintain a controlled release of the loaded drug. The use of polysaccharides in these types of applications is possible only after proper chemical modifications aimed at making them insoluble in aqueous solution. A possible strategy consists in decreasing the hydrophilicity of these materials by introducing hydrophobic groups in their structures, but this often leads to the obtainment of new class of materials, mostly semi-synthetic than naturals and often very different from the original biomaterials. The strategy of election mostly followed by biomaterial scientists in the last 50 years (Hoffmann, 2002) consisted simply in the induction of "junction-zones" between the polymeric chains, inhibiting their dissolution through the formation of permanent or temporary crosslinked networks displaying hydrogel features. These structures maintain almost completely the chemical properties of the original biopolymer comprising their affinity to water giving rise to swollen and not dissolved polymeric scaffolds of natural origins. The maintained hydrophilic character of hydrogels is particularly important in tissue engineering where the overall permeation of nutrients and cellular products into the pores of the gel, determinant for the growth and differentiation of the cells, is determined by the amount of water in the structure (Hoffmann, 2002).

Hydrogels can be chemically or covalently crosslinked and are defined as permanent when the "junction-zones" between the constituting chemical chains are formed by covalent links. If the new bonds are not susceptible to hydrolysis or enzyme recognition the formed hydrogel can be stable indefinitely and not prone to degradation.

The formation of physical or temporary hydrogels is triggered when the "junction-zones" between the polymeric chains are stabilized by weak forces such as electrostatic or hydrophobic interactions. These interactions are reversible, and can be disrupted by changes in physical conditions such as ionic strength, pH, temperature or application of stress. Physical hydrogels are usually not homogeneous, since clusters of molecular entanglements or hydrophobically- or ionically-associated domains can create in-homogeneities (Hoffmann, 2002). This can lead to the formation of hydrogels with weak mechanical properties not suitable for most conventional applications.

Apart from the biological activity displayed by these biomaterials, other important attributes of hydrogels are their mechanical properties and degradation rates that must be tuned according to the final application. The degree of crosslinking along with the chemical

nature of the polymer and of the „junction-zones“ represent the key parameters that mainly affect the above-mentioned properties and can be adjusted according to the addressed applications.

Hydrogels based on polysaccharides are usually characterized by poor mechanical properties due to their impressive water uptake and swelling that lead to the formation of wide opened pore structures that ultimately weakens the scaffold architecture (LaNasa et al., 2010). The presence of charged groups like sulphate and carboxylate in the structure of Ulvan would lead to an even more accentuated absorption of water molecules, hampering the preparation of mechanically stable hydrogels. The strategies for increasing the mechanical properties are several and comprise the preparation of interpenetrating networks with other polymers like polycomplexes formed between oppositely charged polyelectolytes (Hamman, 2010), the preparation of composite hydrogels mixed with inorganic additives (Pavlyuchenko & Ivanchev, 2009) or the use of hydrophobic comonomers as in the preparation of hydrogels by radical crosslinking (Li et al., 2003).

All these strategies strongly affect the chemical nature of the original biopolymers and the properties of the final hydrogels can be very different from those of the native materials comprising their biocompatibility and bioactivity. A strategy to improve the mechanical properties of polysaccharides and in particular Ulvan is based on a proper choice of the nature and amount of “junction-zones” that crosslink the polymer. Indeed both the increase of the crosslinking or the preference for chemical instead of physical crosslinking would positively affect the mechanical properties of the final hydrogels, leading to more compact structures and dimensionally shaped architecture. Both type of crosslinking have been conducted on Ulvan and are worth of mentioning to get a deeper insight on the possible applications and future developments of using this biopolymer in the biomedical fields.

3.1 Ulvan-based physical hydrogels

Ionotropic gelation is a kind of physical crosslinking based on the ability of polyelectrolytes to give hydrogels in the presence of counter-ions. Alginate is a naturally occurring polysaccharide obtained from marine brown algae that spontaneously form reticulated structures in the presence of divalent or polyvalent cations (Patil et al., 2010). The mechanism involves the cooperative interactions of the carboxylate groups of alginate with the polyvalent cations present in solution to form “junction-zones” between the chains that crosslink the matrix in an insoluble polymeric network.

Due to its polyanionic nature Ulvan is expected to show a similar behavior but its gel formation has the unique characteristic of involving borate esters (Haug, 1976). The optimal conditions for the preparations of the hydrogels requires the presence of boric acid and calcium ions at slightly basic conditions (pH 7.5) giving hydrogels with storage modulus of about 250 Pa (Lahaye & Robic, 2007). Higher ion concentrations, different pH, and even phosphate buffering ions are detrimental to the gel.

The mechanism of gel formation is not yet completely unveiled but is proposed to proceed through the formation of borate esters with Ulvan 1,2-diols followed by crosslinking via Ca^{2+} ions (Haug, 1976). Since the gel is thermoreversible the “junction-zones” that crosslink the polymer are thought to involve weak linkages, likely based on labile borate ester groups and ionic interactions easily disrupted by thermal treatments (Lahaye & Robic, 2007). Calcium ions would bridge complexes and/or stabilize the borate esters (Fig. 4a) but also sulphate and carboxylic acid groups were later on proposed to coordinate to Ca^{2+} (Figure 3b,c) (Lahaye & Axelos, 1993) and contribute to the gel formation.

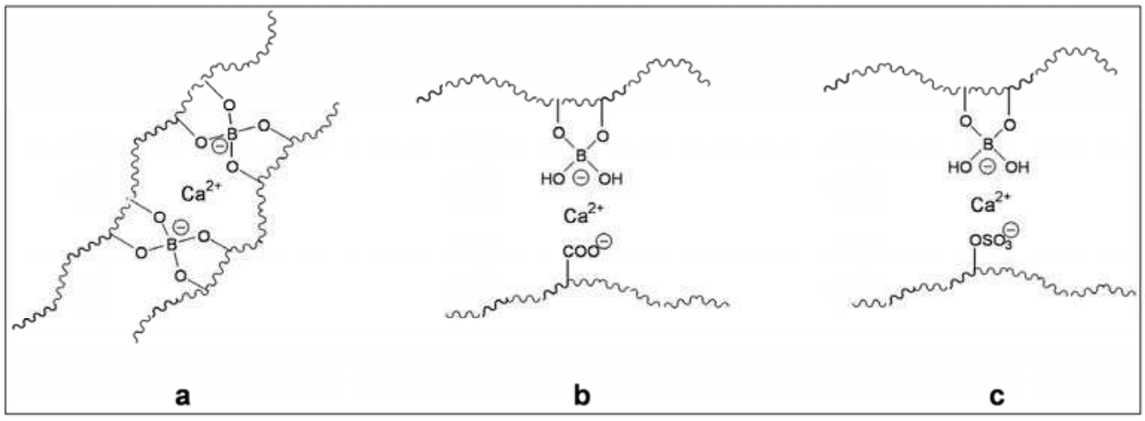

Fig. 4. Proposed mechanisms for the formation of Ulvan-based ionotropic hydrogels through a) Ca^{2+} stabilization of borate esters (Haug, 1976) or the participation of b) carboxylate and c) sulphate groups (Lahaye & Axelos, 1993).

The gel behaviour of Ulvan is different from that of most polysaccharides which usually involve tight junction zones of ordered molecular structures like helices or flat buckled ribbons (Stephen, 1995). Ulvan gel results from the aggregation of bead-like structures interconnected by more hydrophilic polymeric fractions. This behaviour would be undoubtedly favoured by the necklace-type ultrastructure assumed by the polymer in solution, whose formation has been shown to be promoted by ionic interactions (Robic et al, 2009). To this view, the positive role of boric acid can also be related to its reactivity towards the neutrally charged hydroxyl moieties of Ulvan and subsequent substitution with charged borate ester groups, thus contributing to create additional charges on the beads surface of the polysaccharide and favouring their association.

The mechanical properties of these ionotropic hydrogels are usually poor due to their intrinsic weakness and tend to get worse when used in contact with body fluids due to the ion exchange phenomena that occurs between Ca^{2+} that stabilizes the network and the monovalent cations like K^+ and Na^+ present in the physiological liquids (LeRoux et al., 1999). These hydrogels found limited applications in tissue engineering due to their mechanical instability and uncontrolled dissolution in physiological conditions (Atala & Lanza, 2002).

3.2 Ulvan-based chemical hydrogels

In order to overcome the problems related to the mechanical instability of the physically gelled Ulvan and extend the range of their potential applications, the strategy of chemical crosslinking of Ulvan was undertaken (Morelli & Chiellini, 2010).

A smart and relatively innovative technique of obtaining chemically crosslinked hydrogels is represented by their photopolymerization in the presence of photoinitiators using visible or ultraviolet (UV) light.

Photopolymerization is used to convert a liquid monomer or macromer to a hydrogel by free radical polymerization in a fast and controllable manner under ambient or physiological conditions. The mechanism is triggered by visible or UV light that interact with light-sensitive compounds called photoinitiators to create free radicals that can initiate polymerization of species containing suitably reactive groups (typically double bonds).

Photopolymerization has several advantages over conventional polymerization and crosslinking techniques. These include spatial and temporal control over polymerization, fast curing rates at room or physiological temperatures and minimal heat production (Nguyen & West, 2002). Moreover photopolymerization does not require the use of many reactive species, initiator and catalysts usually involved in the conventional chemical crosslinking methods, thus representing a potentially safer technique.

The condition for UV photopolymerization is that polymeric materials need to be conjugated with radically polymerizable groups. In this context, methacryloyl or acryloyl groups, when grafted to the chain backbone via an oxygen or a nitrogen atom usually represent a good candidates for this function, as they work as degradable crosslinks sensitive to either hydrolysis (Benoit et al., 2006) or cell-mediated proteolysis (Mahoney & Anseth, 2006).

The introduction of vinyl or vinylidenic polymerizable groups on Ulvan has been conducted by using several different (meth)acryloyl precursors and conditions (Morelli & Chiellini, 2010) (Figure 5).

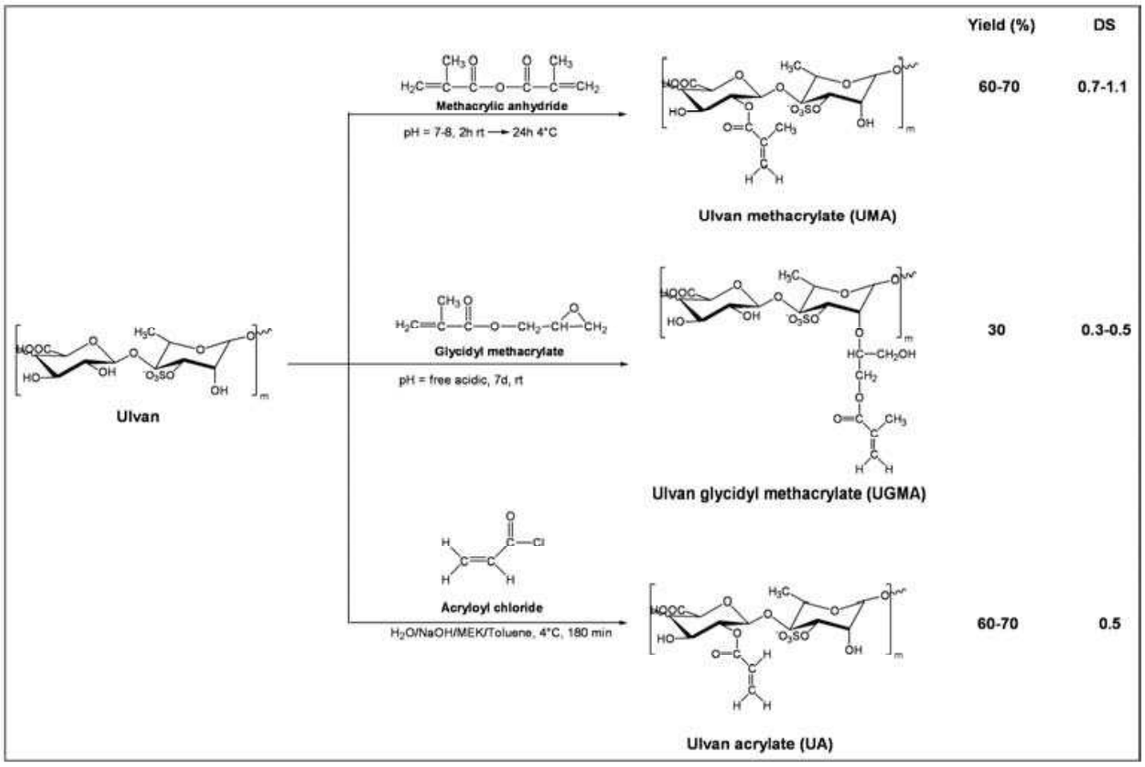

Fig. 5. Reaction of Ulvan with (Meth)acryloyl precursors and relative experimental conditions. Next to every macromer preparation are reported the mean values of the yield (%) of the final products and the degree of substitution (DS) expressed as the mean number of (meth)acryloyl group present in every repeating unit.

The reaction of Ulvan with organic chemical precursors is not straightforward because partially hampered by its insolubility in the common organic solvents. This compels its modification under heterogeneous and not favourable conditions as the ones reported in Figure 5. Also the aggregative behaviour of Ulvan in aqueous solutions limits its "reactive

surface" available for modifications and this may be the cause for the low degree of (meth)acryloyl substitution usually found on the final macromers.
The amount of UV polymerizable unsaturated groups introduced onto the polysaccharide would definitively affect the physical properties of the final hydrogels because it determines the number of "junction-zones" that crosslink the linear polymer chains. Both the substitution of the polar hydroxyl groups with the hydrophobic (meth)acryloyl moieties and the increase of the number of crosslinks inside the hydrogel structures would determine a minor absorption of water molecules with a consequent improvement in the mechanical properties of the hydrogels (Anseth et al., 1996). To this view the mean number of crosslinkable groups present in every repeating unit of Ulvan – expressed as substitution degree (SD) - represents a key parameter to be evaluated.
The calculation of the substitution degree (SD) of the macromers obtained by the different chemical routes reported in Figure 5, has been performed by ^{1}HNMR analyses (Figure. 6).

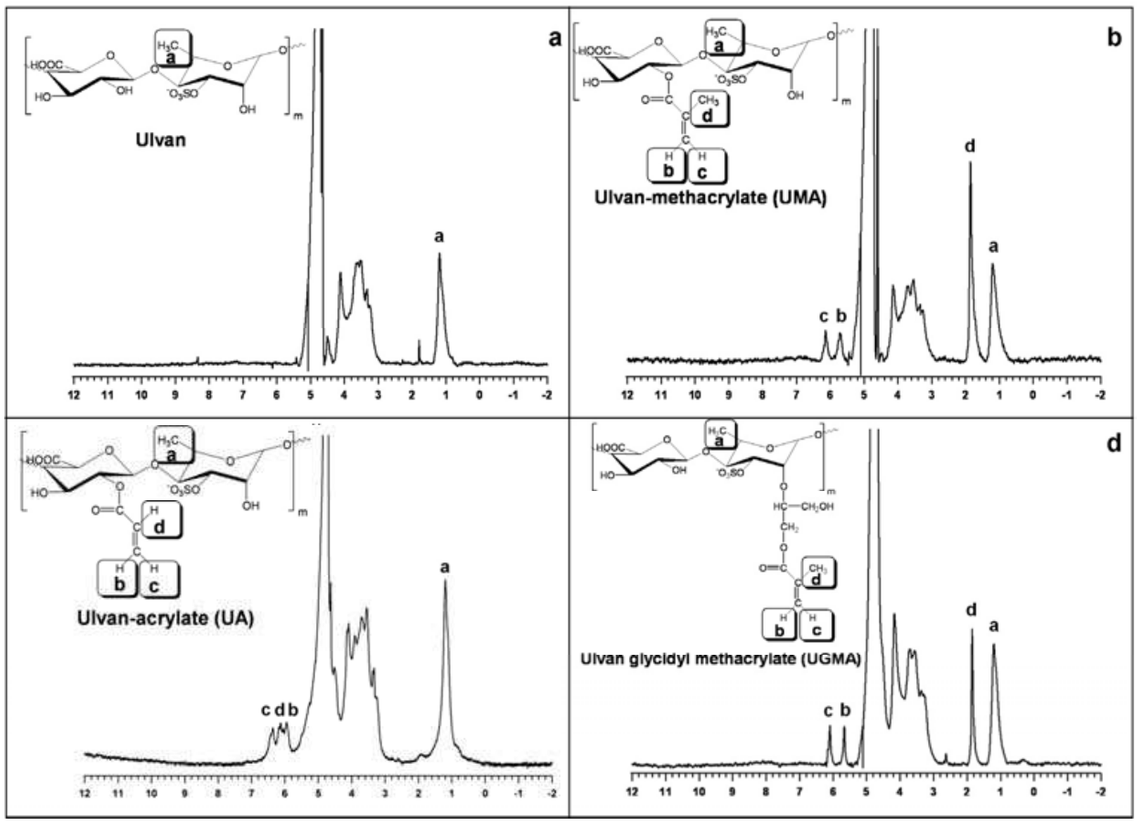

Fig. 6. ^{1}HNMR spectra in D_2O of : a) Ulvan, b) Ulvan-methacrylate (UMA), c) Ulvan-acrylate (UA). d) Ulvan-glycidyl methacrylate (UGMA). Chemical structures of the main disaccharide repeating units of the polysaccharides are reported together with the relative peak assignment as highlighted in the small boxes.

The SD has been calculated by comparing the peak areas relative to the vinyl protons of the introduced (meth)acryloyl groups (Fig. 6b-d) with the peak area relative to the methyl group of the rhamnose present in the native Ulvan (Fig. 6a). The SD values reported in Figure 5 represent only a rough estimation of the actual values because the chemical structure of the

repeating unit of Ulvan used for the calculations (Fig. 6a) can not be considered univocal (Lahaye & Robic, 2007).
The most effective procedure for the preparation of macromers has been shown to utilize a large excess of methacrylic anhydride in slightly basic conditions for 24 hours at 4°C (Fig. 5). A fairly large amount of reactive is required because of the competitive hydrolysis that spoils the methacryloyl precursor during the reaction. The formation of the methacryloyl derivative of Ulvan was further confirmed by FT-IR analysis (Fig. 7).

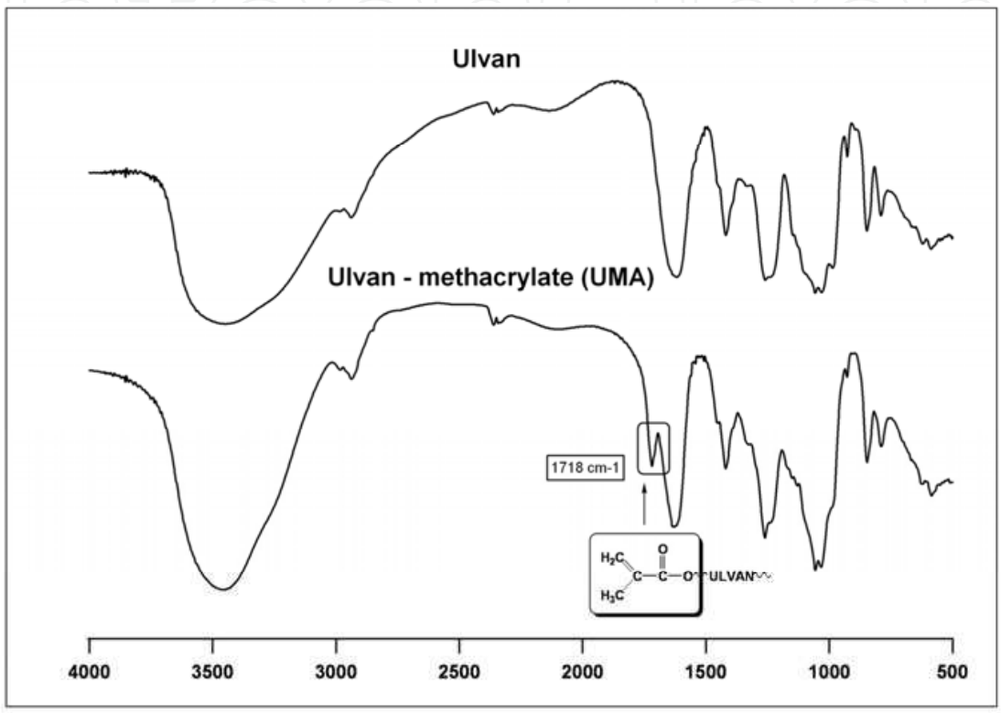

Fig. 7. FT-IR spectra of Ulvan and Ulvan methacrylate (UMA) macromer with the structure of the conjugated methacryloyl group and the relative absorption frequency reported in small boxes.

The spectra of the native Ulvan and the Ulvan methacrylate derivative are completely overlapping except for the peak at 1718 cm^{-1} likely attributable to the presence of an α,β-unsaturated carboxylic ester.
The preparation of hydrogels has been carried out by using a small amount of a cytocompatible photoinitiator – IRGACURE ® 2959 – and exposing the polymeric solution to UV light – 365 nm – at short irradiation times. The mechanism of the covalent crosslinking between the polymeric chains involves the radical polymerization of the conjugated (meth)acryloyl groups and the formation of degradable carboxylic ester based "junction-zones" that act as crosslink moieties (Figure 8).
The crosslinking degree (CD) of Ulvan macromers have been evaluated by ^{1}HNMR analyses of the solutions before and after definite times of irradiation by monitoring the peak areas of the reacting unsaturated protons (**b** and **c**, Figure 9). This technique has been applied to UMA because the preparation of this macromer proved to be the most effective in terms of yield and SD.

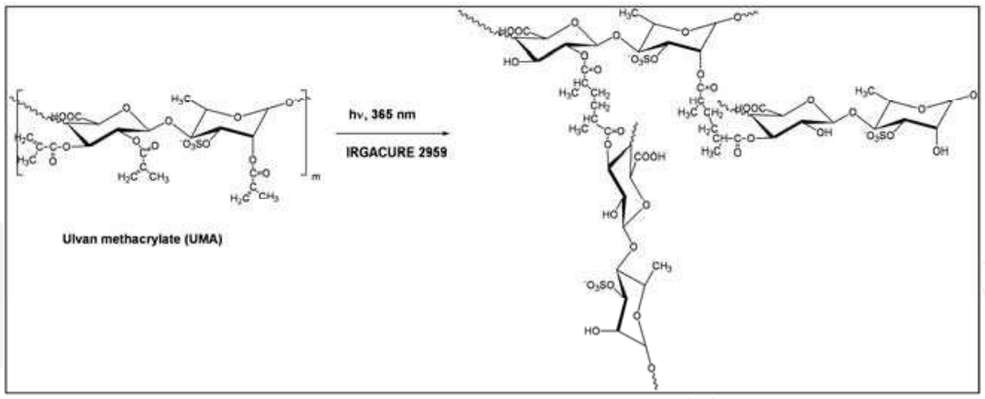

Fig. 8. Photopolymerization of Ulvan methacrylate under UV irradiation – 365 nm – in presence of a photoinitiator.

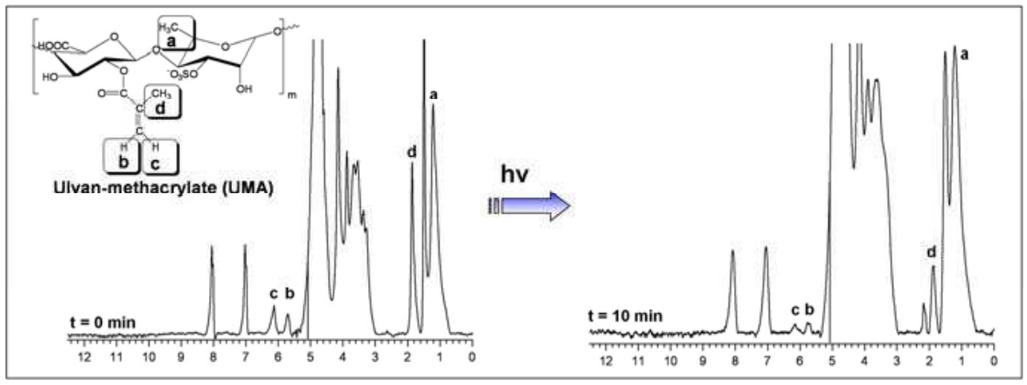

Fig. 9. ^{1}HNMR spectra of Ulvan-methacrylate (UMA) before and after 10 min of UV irradiation in the presence of IRGACURE® 2959 as photoinitiator. Chemical structures of the main disaccharide repeating units of the macromer is reported together with the relative peak assignment as highlighted in the small boxe.

The exposure of Ulvan macromers to UV light Leads definitely to the formation of hydrogels but the photopolymerization is not complete even after 10 minutes of irradiation as demonstrated by the residual peak of the unreacted double bond protons **c** and **b** in the ^{1}HNMR spectrum. The degree of crosslinking has been monitored at fixed irradiation times and showed that half (meth)acryloyl groups are polymerized after 5 minutes of UV curing (Table 1).

UV exposure time (min)	CD (%)
1	22.5
2	31.5
3	38.0
5	51.0
10	68.5

Table 1. Crosslinking degree (CD) for UMA having SD = 1 under UV irradiation (365 nm, 8 mW $\cdot$cm^{-2}) as a function of exposure time.

The uncomplete crosslinking of Ulvan macromers under UV exposure could be ascribed to both the nature and the morphology assumed by this polysaccharide in solution. Indeed the antioxidant activity of Ulvan could reduce the rate of radical polymerization of the macromers by quenching the radicals formed during the UV irradiation. Moreover the aggregative behaviour of Ulvan in aqueous solution reduces the amount of (meth)acryloyl groups available for polymerization thus partially inhibiting the crosslinking.

The property of retaining water represents a key parameter for evaluating the quality of a hydrogel and its potential use for biomedical applications because it usually affects its permeability, biocompatibility and rate of degradation. The swelling ability of hydrogels could also provide information about their mechanical stability and chemical and physical properties, since the degree of water uptake is related both to the chemical nature and to the physical structure of the polymeric network (Qi et al., 2004). It is known, for example, that gels exhibiting a larger pore structure – likely due to a lower degree of crosslinking – have poor mechanical strength and higher swelling ratios (Anseth et al., 1996).

The swelling ability of hydrogels is usually quantified by measuring their Swelling Degree % (SD%) taken as the ratio (%) between the weight of the swollen hydrogel to that of the dried sample. The swelling degrees of the prepared Ulvan hydrogels have been carried out in phosphate buffer solutions (pH 7.4) and their behaviour was recorded during 7 days of immersion (Fig. 10).

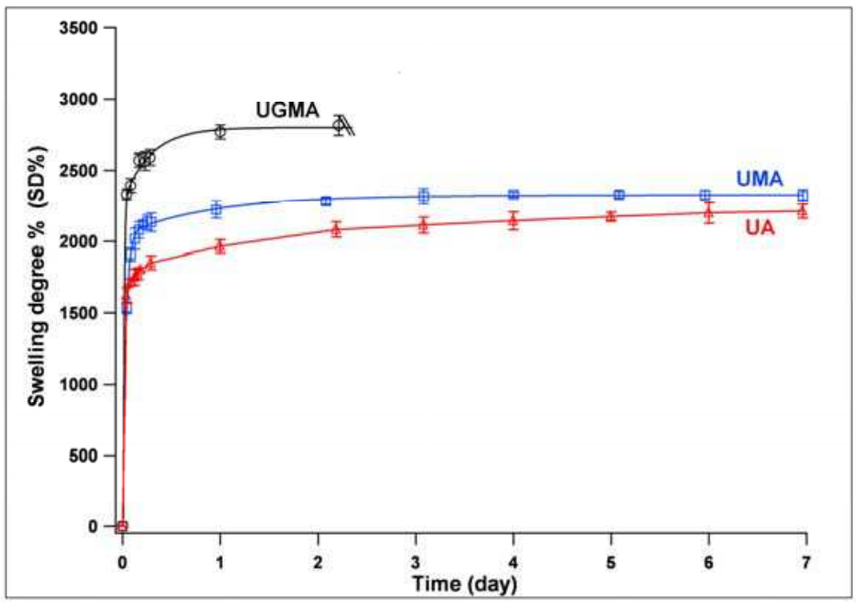

Fig. 10. SD% in PBS buffer solution (0.1 M, pH 7.4) of UV crosslinked (365 nm, approximately 8 $mW \cdot cm^{-2}$) UMA and UGMA hydrogels as a function of time.

Pictures of the swollen scaffolds taken after 2 days of immersion in phosphate buffer saline (PBS) at pH 7.4, showed that the Ulvan methacrylate (UMA) hydrogels proved to be most stable in terms of texture and mechanical properties (Figure. 11).

The swelling degree experiments of UGMA-based samples were stopped after 2 days of immersion since the hydrogels were no longer coherent and hence not easy to handle. This

behaviour could be interpreted by considering the low degree of substitution typically obtained with this type of macromer (Figure 5) and subsequent low degree of crosslinking. The final hydrogels resulted more hydrophilic, as demonstrated by the highest swelling degree values (SD%) obtained, and less crosslinked thus leading to network with weak mechanical properties.

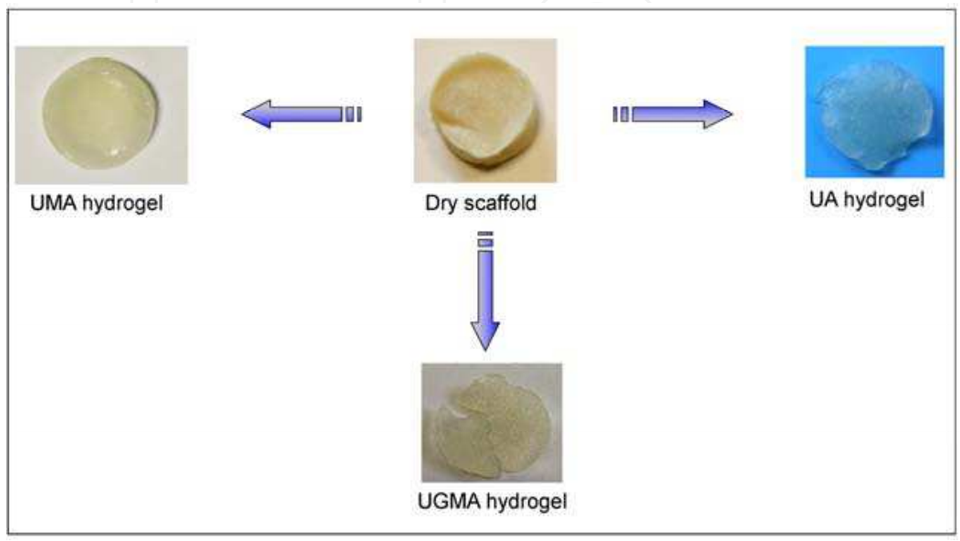

Fig. 11. Pictures of the swollen hydrogels obtained after 2 days of immersion in the swelling medium (phosphate buffer, 0.1M, pH 7.4).

The swelling degree values obtained with UMA- and UA-based macromers resulted to be similar thus indicating a similar density of crosslinking inside their structure. The interpretation of these datas are not straightforward since UMA-based hydrogels were expected to be less hydrophilic and more crosslinked due to the higher amount of polymerizable group contained in their structure. Indeed the final texture of the UMA-based hydrogels indicated a better mechanical stability in respect to the other type of hydrogels. The unexpected lower SD (%) values obtained with UA-based hydrogels could be explained by the loss of material during the swelling experiments.

4. Conclusion

Ulvan, a sulphated polysaccharide of algal origin, is worth of deeper attention for its potential use in technological and industrial-related applications. The exploitation of this abundant and renewable resource could represent an advantageous alternative approach to the use of fully synthetic materials based on fossil fuel feedstock.

In particular Ulvan represents an intriguing candidate material for biomedical applications due to its intrinsic beneficial biological activities and the possibility of easily modifying its structure according to the envisaged application. Its chemical structure similar to that of natural glycosaminoglycans such as chondroitin sulphate and hyaluronic acid make Ulvan an attractive candidate for their substitution or use in related applications.

In order to be employed in biomedical applications such as tissue engineering, regenerative medicine and drug delivery, Ulvan needs to be converted into an insoluble material under physiological conditions and to have mechanical properties suitable for the end application. The preparation of physically crosslinked Ulvan hydrogels has been reported since long times, but their weak mechanical properties and uncontrolled dissolution in presence of physiological fluids make them unsuitable for biomedical uses, where a scaffolding role is required

A novel method for covalent crosslinking of Ulvan through the UV mediated radical polymerization of activated macromers by double bond conjugated moieties, revealed to be promising in the preparation of chemically crosslinked Ulvan hydrogels. The conjugation of methacryloyl group to Ulvan through the reaction with methacrylic anhydride under slightly basic conditions gave the best results in terms of product yield and substitution degree. The hydrogels obtained after their exposure to UV light seemed to be very stable in physiological conditions.

The crosslinking of the Ulvan macromer precursors is usually not complete because is hampered both by its aggregative behaviour in solution that limits the availability of the (meth)acryloyl groups and very presumably by the radical quenching activity of the polysaccharide during the UV exposure thus negatively affecting the mechanical properties of the final hydrogels. Nevertheless the antioxidant activity of Ulvan could make this material a good candidate as a matrix for cell encapsulation due to the possible protection against the radicals produced during UV crosslinking (Fedorovich et al., 2009). The use of these materials as a base for cytocompatible scaffolds is also promoted by the softness related to partial crosslinking of these macromers, since it is known that cell spreading within hydrogels is influenced by matrix stiffness and soft matrices interestingly are expected to promote cell spreading (Liu & Chan-Park, 2009).

Moreover the possibility of preparing Ulvan based hydrogels by a straightforward technique such as UV crosslinking makes the use of Ulvan in biomedical fields even more attractive. Indeed UV photopolymerization allows the spatial and temporal control over the crosslinking and the fabrication of hydrogels in situ with the possibility of forming complex architectures that adhere and conform to tissue structure.

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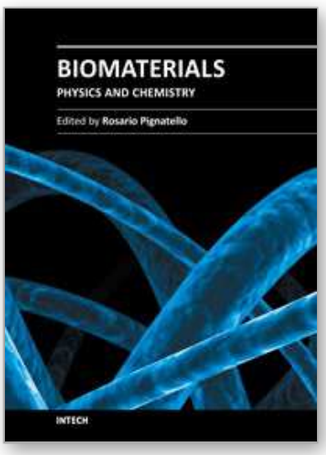

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These contribution books collect reviews and original articles from eminent experts working in the interdisciplinary arena of biomaterial development and use. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentialities of different synthetic and engineered biomaterials. Contributions were selected not based on a direct market or clinical interest, but based on results coming from very fundamental studies. This too will allow to gain a more general view of what and how the various biomaterials can do and work for, along with the methodologies necessary to design, develop and characterize them, without the restrictions necessarily imposed by industrial or profit concerns. The chapters have been arranged to give readers an organized view of this research area. In particular, this book contains 25 chapters related to recent researches on new and known materials, with a particular attention to their physical, mechanical and chemical characterization, along with biocompatibility and hystopathological studies. Readers will be guided inside the range of disciplines and design methodologies used to develope biomaterials possessing the physical and biological properties needed for specific medical and clinical applications.

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