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Processing ulvan into 2D structures: Cross-linked ulvan membranes as new biomaterials for drug delivery applications

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ABSTRACT

The polysaccharide ulvan, composed of sulphated rhamnose, glucoronic and iduronic acids was used to produce polymeric membranes by solvent casting. As ulvan is soluble in water, a cross-linking step was necessary to render the membrane insoluble in water and stable at physiological conditions. Cross-linked ulvan membranes were characterized by FTIR, SEM, swelling behaviour was investigated and the mechanical performance assessed by quasi-static tensile testing. Furthermore, the ability and mechanism of sustained release of a model drug from ulvan membranes was investigated. Produced membranes revealed remarkable ability to uptake water (up to ~1800% of its initial dry weight) and increased mechanical performance (1.76 MPa) related with cross-linking. On the other hand, medicated ulvan dressings demonstrate the potential as drug delivery devices. Using a model drug we have observed an initial steady release of the drug – of nearly 49% – followed by slower and sustained release up to 14 days. The properties of ulvan membranes herein revealed suggest a great potential of this natural sulphated polysaccharide as a wound dressing.

1. Introduction

Wound dressings are widely applied for the treatment of different wounds such as burns, trauma and diabetic ulcers amongst others (Boateng et al., 2008; Lim et al., 2010). Currently commercial available wound dressings can be made of a wide range of materials including polyurethane (Ex: PolyMem®) or gelable polysaccharides, such as starch (Ex: Iodosorb®) and carboxymethylcellulose (Ex: Aquacel®) (Boateng et al., 2008; Lim et al., 2010; Mohajer and Cohen, 2006; Yoo and Kim, 2008). The abundance of available dressings is largely related with the demand of an effective management of the different types of wounds. Furthermore, the complexity of the wound healing process per se may require the use of several types of dressings, including medicated dressings (Boateng et al., 2008). These in particular are meant to provide a sustained delivery of a therapeutic agent for a desired period of time providing aid in the treatment, management (including pain) and eventual healing of a wound (Boateng et al., 2008).

Different materials are chosen to produce these systems, ranging from natural to purely synthetic polymers. In this context, natural origin materials offer advantages with respect to biodegradability, biocompatibility and wide availability (d'Ayala et al., 2008). Amongst the diversity of proposed nature origin biomaterials, marine derived polysaccharides are already being investigated for wound dressing applications, namely chitosan and/or alginate (Meng et al., 2010; Silva et al., 2004, 2005). In the present study, we have focused our research on ulvan. This rather unexploited polysaccharide is anionic, water soluble, sulphated and semi-crystalline (Alves et al., 2010). Green algae, namely *Ulva* the common sea lettuce, are frequently involved in algal proliferation in eutrophicated coastal and lagoon waters (Morand and Briand, 1996). This biomass has diminished associated value and is either employed into compost production or simply dumped (Lahaye and Axelos, 1993). Ways to use and add value to this biomass could be based on specific properties of their cell-wall polysaccharides and their application in high technological areas of knowledge, including the biomedical field. The exploitation of ulvan as an alternative to different synthetic or animal origin polymers would benefit of its algal origin, bioavailability and low expected production costs as well as associated biological activities. In fact, unmodified ulvan possesses interesting properties that can be directed towards highly demanding application areas like the pharmaceutical or food industry (Bocanegra et al., 2009; Lahaye, 1991; Smit, 2004). However, some degree of modification of polysaccharides is

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many times required in order to control and fine tune its final properties to particular applications (Barbosa et al., 2005; Malafaya et al., 2007; Shoichet, 2009). Obtained polysaccharide derivatives may possess desirable properties that can widen their array of applications. In the particular case of ulvan, the study of polysaccharide modification is largely related with the production of biomaterials and reported work in this area relates to functionalization of this polysaccharide (Morelli and Chiellini, 2010). In this pioneer work, Morelli and Chiellini (2010) report the modification of ulvan by grafting with methacryloyl groups in order to produce stable and biodegradable hydrogels aimed for biomedical applications.

Nevertheless, application studies based on ulvan are scarce, especially concerning polysaccharide modification, processing and biomaterial design. Given the peculiar nature of this polysaccharide and its inherent biological effects, it is easy to envisage focused applications based on these properties. Ulvan's biological properties can be related to its sulphation degree, but also with its particular sugar composition (Pomin and Mourão, 2008; Yang and Zhang, 2009). For instance, the ubiquitous occurrence of rhamnose in this algal polysaccharide can be considered as an advantageous characteristic, particularly for the treatment of skin pathologies (Andrès et al., 2006; Faury et al., 2011). In general, rhamnose-rich polysaccharides demonstrate anti-inflammatory properties, diminish skin bacterial adhesion, protect it from UV-induced and age-related injure and stimulate cellular proliferation and collagen biosynthesis (Andrès et al., 2006; Faury et al., 2011). Furthermore, ulvan has been described as a heparinoid agent (El-Baky et al., 2009; Harada and Maeda, 1998; Mao et al., 2006). This heparin-like character positions ulvan as a strategic choice for wound management applications, as heparin, heparin derivatives and heparin-like compounds are known to exert positive effects on chronic wounds (Hehenberger et al., 1998). In fact, heparin is often applied in the treatment of wounds, as this sulphated polysaccharide plays a pivotal role in the wound healing process, either by promoting fibroblast proliferation, inhibition of thrombin generation, improvement of fibrinolytic functions, or stimulation of heparin sulphate synthesis (Hehenberger et al., 1998; Kalani et al., 2007; Rullan et al., 2008; Trindade et al., 2008). Within this context, the sulphated polysaccharide ulvan can be considered for applications in wound management, particularly as a wound dressing. Preparation of polymeric membranes to be applied as wound dressings can be performed by solvent casting, as this is a widespread method to process polymers into membranes (Silva et al., 2004, 2005). Besides ease of preparation and low costs associated with this manufacturing method, it can be an effective way to incorporate a therapeutic agent into the membrane, by mixing it with the polymer prior to casting (Grassi and Grassi, 2005). After evaporation of the solvent, a combined polymer-drug membrane (medicated dressing) is obtained (Zilberman, 2005).

As the fundamental knowledge about ulvan evolves, the development of various modification and processing routes becomes decisive to its progress towards highly demanding areas. Hence the main objective of the present research work is to evaluate the filmogenic properties of ulvan and its feasibility to be applied as a wound dressing, especially as a drug delivery system.

2. Experimental procedure

2.1. Materials

Ulvan was extracted from green algae as described elsewhere (Alves et al., 2010). 1,4-Butanediol diglycidyl ether (BDDE) and dexamethasone were provided by Sigma.

2.2. Cross-linking ulvan

Ulvan was mixed with BDDE (1:5 molar ratio), in an alkaline media (sodium hydroxide, 40 mM). The cross-linking reaction was allowed to proceed for 180 min, at 50 °C. Cross-linked ulvan powder was exhaustively washed with water and acetone to remove any residual cross-linker. The obtained dried powder was further used to produce cross-linked ulvan membranes.

2.3. Membrane preparation

To prepare the polymeric membranes, cross-linked ulvan was dispersed in water (1%, w/v) and the solution was homogenized with an UltraTurrax apparatus. Ulvan membranes were prepared by casting ulvan solution on Petri dishes, followed by solvent evaporation, at 50 °C, in a vacuum oven.

Ulvan membranes impregnated with dexamethasone were prepared as described above, except that dexamethasone was dissolved in acetone and added to ulvan solution, with a final concentration of 15% (w/w), prior to solvent casting. In the present study, dexamethasone, a steroid anti-inflammatory drug, was used as a model therapeutic agent. Non cross-linked ulvan membranes were prepared as described to study the cross-linking mechanism and the influence of cross-linking on the overall mechanical performance. The final appearance of ulvan membranes, loaded or not with dexamethasone, is a homogeneous and transparent yellowish film.

2.4. Fourier transform infrared spectroscopy (FTIR)

Chemical modifications introduced by the cross-linking reaction were investigated by FTIR. The infrared spectra were recorded on a spectrophotometer (IR-Prestige-21, Shimadzu, Japan), controlled by IRsolution software. All spectra were averaged on 32 scans in the range of 600–4400 cm^{-1} with a resolution of 4 cm^{-1}.

2.5. Scanning electron microscopy (SEM)

Surface morphology of the produced membranes was analyzed using a Leica Cambridge S-360 scanning electron microscope (SEM) (Leica Cambridge). All specimens were pre-coated with a conductive layer of sputtered gold.

2.6. Mechanical properties testing

Ulvan membranes were subjected to tensile tests to evaluate the effect of cross-linking over the mechanical properties and to determine the mechanical performance of these membranes. Membranes with 500 μm in thickness were cut into 10-mm-long specimens and tensile tests, in dry state, were performed using a Universal Testing Machine (Instron 4505), with a load cell of 1 kN, gauge length of 10 mm and cross-head speed of 5 mm/min was used up to rupture of the membrane. A minimum of five specimens were tested for each sample (the values reported are the average of those results). These tests allowed the determination of different mechanical properties defined as follows: tensile modulus defined as the slope of the straight line obtained by linear regression of the stress–strain curve in the near elastic region of the material (between 0 and 1.0% strain); ultimate tensile strength (referred as tensile strength), defined as the maximum tensile stress developed in the material during the tensile test and strain at break point (referred as tensile strain), defined as the maximum strain of the material, i.e. elongation at the failure point of the material.

2.7. Water uptake study

The hydration degree of ulvan polymeric membranes was assessed over a period of 14 days. Five specimens (previously weighed) were immersed in phosphate buffered saline (PBS, pH 7.4) and incubated at 37 °C for 1, 3, 7 and 14 days. After each defined period of time, the specimens were removed from the PBS solution, gently blotted with a paper filter and the weight was again measured. The water uptake was calculated by the following equation:

$$\text{Water uptake}\,(\%) = [(w_s - w_i)/w_i] \times 100$$

where w_i is the initial weight of the specimen before immersion and w_s is the wet mass of the specimen at time t (days) after being removed from the solution.

2.8. In vitro dexamethasone release profile

Five specimens of ulvan cross-linked membranes loaded with dexamethasone were immersed in PBS (pH 7.4) and incubated at 37 °C for a period of 14 days. The release of dexamethasone was periodically monitored by extracting 500 μl aliquots and replenishing with 500 μl of PBS, in predetermined time intervals. The concentration of dexamethasone was determined by UV–vis spectroscopy at 242 nm (Shimazu UV 1601). The results presented are an average of five measurements.

2.9. Mathematical modelling of dexamethasone release from ulvan membranes

To better understand the mechanisms of dexamethasone release from ulvan membranes the power law (or Peppas equation) was applied to the experimental data (Siepmann and Peppas, 2001; Siepmann and Siepmann, 2008). This particular model was chosen as it accounts for both drug diffusion and matrix swelling. Power law is a simple empirical equation, which describes a linear relationship between logarithm of the amount of drug released and logarithm of time, up to 60% of the maximum drug released:

$$\frac{M_t}{M_\infty} = kt^n$$

M_t/M_∞ is related with the fractional drug release, k is the kinetic constant, t refers to the release time and n is the diffusional exponent that is related to the drug transport mechanism. For a thin polymeric membrane, the drug release mechanism is Fickian diffusion when $n = 0.5$, $n = 1$ Case II transport occurs leading to zero-order release and if the value of n is between 0.5 and 1, anomalous transport is observed (Duarte et al., 2009; Siepmann and Peppas, 2001; Siepmann and Siepmann, 2008).

3. Results and discussion

In the present research work, the development of cross-linked ulvan membranes is described and their use for local delivery of drugs, as medicated dressings, is proposed. In this type of applications, stability in physiological conditions constitutes a desired property. As unmodified ulvan is readily dissolved in water cross-linking plays a decisive role in tuning the properties of the final polymeric matrix.

BDDE is one of the most frequently used homobifunctional epoxide reagent to cross-link polysaccharides. It is considered cytocompatible which is of extreme importance when we are focusing on biomedical applications (Zawko et al., 2009). The epoxide functionalities of BDDE are able to react with different groups like hydroxyls, amines or thiol groups (Zeeman et al., 2000; Zhao et al., 2002). Polyepoxide compounds have already been successfully used to cross-link collagen, gelatine and hyaluronic acid (Zawko et al., 2009; Zeeman et al., 2000). In fact BDDE is the cross-linking agent used to cross-link many commercially available products, namely in aesthetics, such as dermal fillers like Juvederm® or Restylane® (Baumann, 2009).

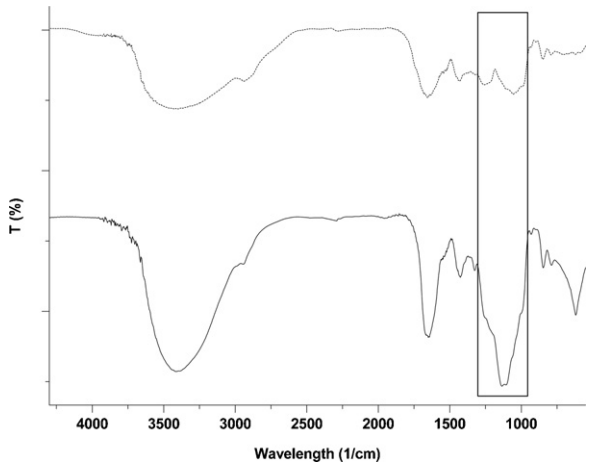


Fig. 1. FTIR spectra (straight line: ulvan; dash line: cross-linked ulvan).

For all the above reasons, chemical modification of ulvan was performed via cross-linking with BDDE. In order to try to identify the bonds responsible for the cross-linking of ulvan with BDDE and understand the mechanism of reaction, FTIR spectroscopy was performed on the processed membranes, with and without cross-linking. By analyzing the FTIR spectra of Fig. 1, it is possible to identify different characteristic peaks of ulvan: stretching (st) vibration of polymeric hydroxyl groups at 3500–3200 cm^{-1}; it is also possible to detect the presence of carboxyl groups by a band at 1650 cm^{-1}, the asymmetric stretching of the ether glycoside bridge and the symmetric and asymmetric stretching of the ether sulphate group in the range of 1315–1220 cm^{-1} and 1140–1050 cm^{-1} (Alves et al., 2010). By comparing FTIR results before and after cross-linking, it is not possible to detect the formation of novel bonds, which could have been introduced during the cross-linking reaction. There are several groups present in the region of 900–1250 cm^{-1} (highlighted in Fig. 1), characteristic of native ulvan. However, after cross-linking, the signal around 1090 cm^{-1} is markedly decreased. The disappearance of this signal may be due to the consumption of —OH needed for the cross-linking reaction to occur, as this band can be attributed to CH—OH (st).

Epoxide groups are able to react with different nucleophiles, including amines, thyols and hydroxyl groups, present within the polymer backbone, yielding stable covalent bonds (Hermanson, 2008; Malson, 1987; Zhao et al., 2002). Reaction with different nucleophyles, in appropriate experimental conditions, will result in different end bonds. For example, efficient coupling of epoxide moieties with hydroxyls will occur in an alkaline media, at high pH, yielding stable ether bonds; primary amine groups react with epoxide groups at moderate alkaline pH, resulting in secondary amines or sulphydryl groups yield thioether bonds in a reaction with epoxides at close to neutral pH (Hermanson, 2008). In this scheme, given that reaction of ulvan with BDDE occurs in an alkaline moiety, at high pH, and considering the apparent consumption of hydroxyl groups, as demonstrated by FTIR, ether bonds are expected to be formed. A reaction mechanism is proposed and illustrated in Fig. 2. Reaction is thought to occur via ulvan's hydroxyl groups that are able to react with the epoxide moiety of BDDE in alkaline media, yielding a stable ether bond. Cross-linking may be complete, bringing together two polysaccharidic chains via ether

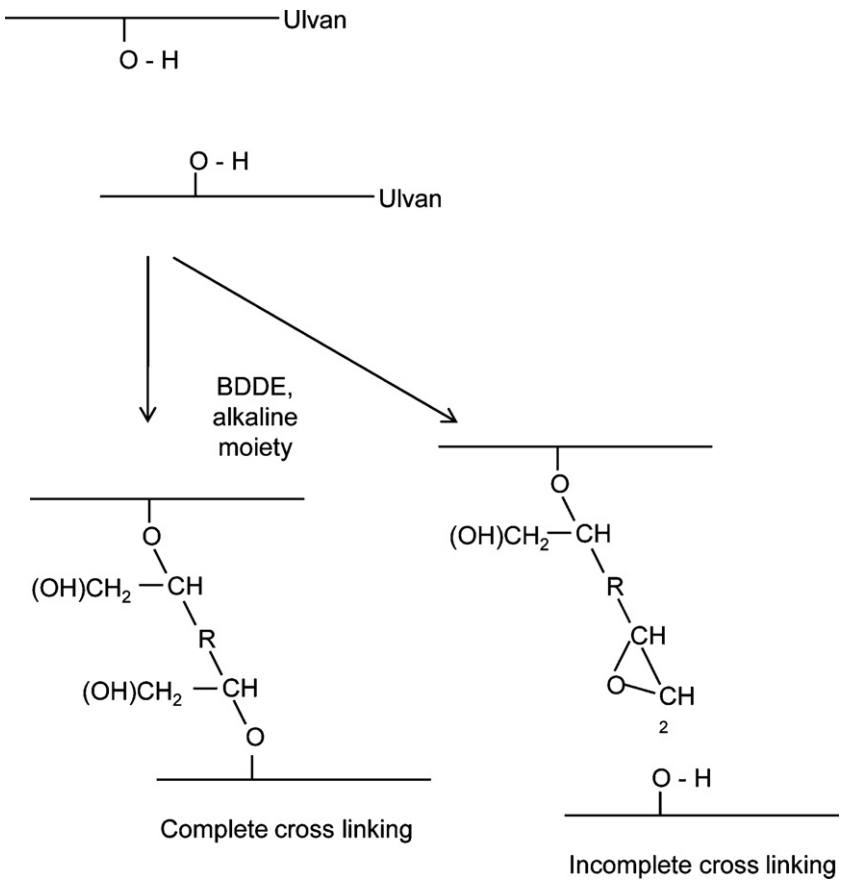


Fig. 2. Proposed cross-linking mechanism of ulvan polysaccharide with BDDE.

bonds, or incomplete, with epoxide pendant groups (Zeeman et al., 2000). Given the bifunctional nature of BDDE, pendant epoxide groups may be expected, due to limited reagent concentration or due to absence of vicinal functional groups available to react with the epoxide (Zeeman et al., 2000).

Surface studies by SEM revealed that ulvan membranes are non porous and their surface is homogeneous, presenting some degree of roughness (Table 1).

As ulvan membranes are envisaged for applications as dressings, they are required to be resilient and stress resistant to be able to cope with the stresses exerted during manual manipulation or by surrounding tissue, and this endorses the importance of mechanical behaviour on this particular applications (Boateng et al., 2008). To better understand the influence of cross-linking on the mechanical performance of the designed membranes, a comparison between non cross-linked membranes and cross-linked membranes was performed. When comparing the results obtained for both types of ulvan membranes, it is notorious the striking effect of cross-linking on the mechanical performance of ulvan membranes; in general, these properties can be improved by cross-linking (LeRoux et al., 1999; Wu et al., 2010). Tensile strength and modulus were significantly improved by the cross-linking reaction (Table 2). Membranes without cross-linking have a tensile strength of 4.7 kPa and a tensile modulus of 580 kPa. Cross-linking ulvan yielded membranes with increased tensile strength and tensile modulus, causing the tensile strength to increase to 44 kPa and the tensile modulus to increase by 203%. However, this made ulvan membranes less ductile, as it can be seen by the decrease in tensile strain.

In order to evaluate the swelling behaviour of ulvan membranes they were placed in a buffer solution, at 37 °C. Fig. 3 presents the profile of the water-uptake capability of cross-linked ulvan membranes as a function of time and demonstrates that ulvan membranes possess significant water uptake ability, showing a maximum peak after 14 days in PBS (~1800%). The ability to uptake water must be one of the most remarkable and defining properties of the produced ulvan membranes. Maintenance of moisture in a wound is considered a critical aspect of wound management as a

Table 1
Surface topography of produced ulvan membranes.

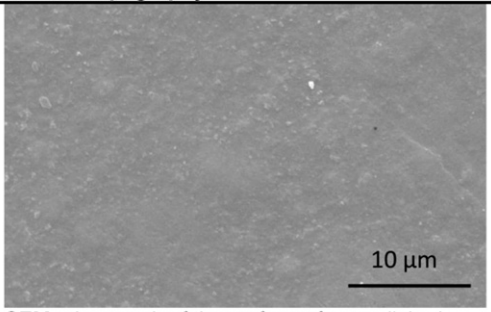
Surface topography

SEM micrograph of the surface of cross-linked ulvan membrane (Magnification 3000x)

Table 2
The mechanical properties obtained from the tensile curves are presented in the following table.

Ulvan membrane	Tensile modulus (kPa)	Tensile strength (kPa)	Tensile strain (%)
Without cross-linking	580.0 ± 213.00	4.7 ± 1.89	26.4 ± 10.01
With cross-linking	1760.0 ± 284.00	44.0 ± 12.00	15.2 ± 7.66

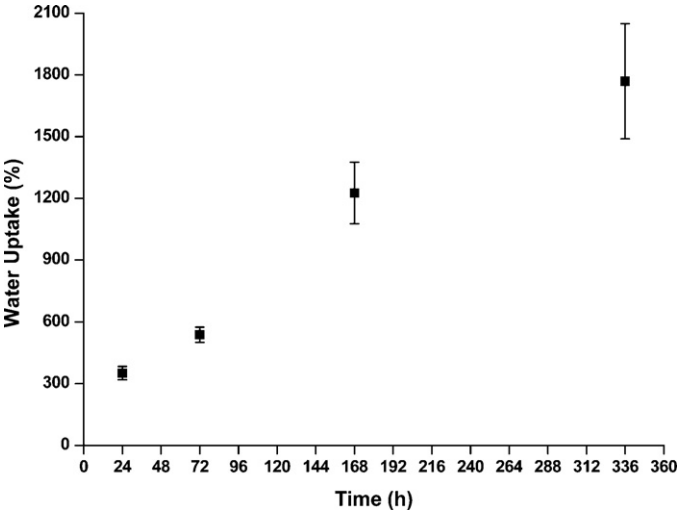


Fig. 3. Percentage of water uptake by cross-linked ulvan membranes along time. Results are presented as mean ± SD of *n* samples per time point.

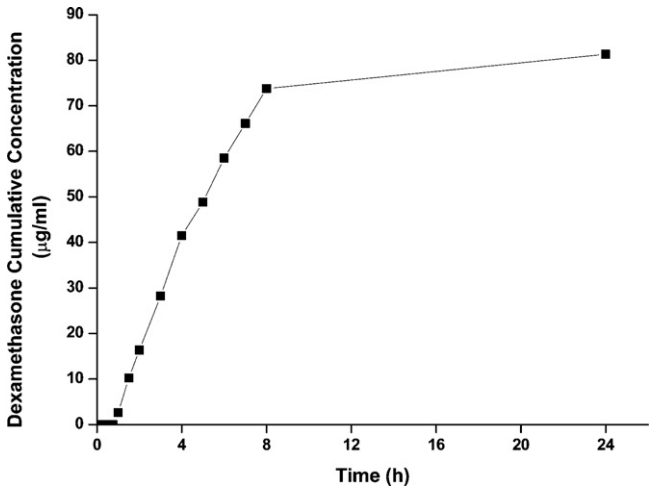


Fig. 5. Detail of dexamethasone concentration release profile from ulvan membranes. Results are presented as mean ± SD of *n* samples per time point.

moist environment may favour the healing process through prevention of desiccation and cell death, stimulation of cell migration, angiogenesis, connective tissue generation and autolysis (Boateng et al., 2008; Paul and Sharma, 2004). Furthermore, if high water absorption from exudate wounds is desired, ulvan membranes' hydration ability assumes a pivotal role (Boateng et al., 2008). In this context, the water uptake demonstrated by ulvan membranes becomes relevant and supports their feasibility to be applied as wound dressings.

On the other hand, this knowledge becomes relevant particularly when these membranes are aimed for drug delivery applications. In the present study, cross-linked ulvan membranes were loaded with a therapeutic model drug, dexamethasone, so as to evaluate their ability to be further used as medicated wound dressings. From Fig. 4, and in more detail in Fig. 5, a steady release of dexamethasone is observed for 8 h. At this point, 49% of drug has been release from the prepared membranes. Afterwards, a slower release of the drug is detected and at day 14 around 72% of dexamethasone has been released from ulvan membranes.

There are several factors that influence the release of a drug from a matrix, including diffusion of the drug out of the matrix and the water uptake by the polymeric matrix (Duarte et al., 2009).

One factor is mostly related with the drug itself and the other is related with the polymer and the polymer network. Given the water uptake ability of ulvan membranes, we can hypothesize that the influence of ulvan's swelling on the release of the drug is significant. As ulvan membranes are regarded as a non-porous system, drug molecules are released through the network meshes within the polymeric phase (Grassi and Grassi, 2005). This theoretical scenario is supported by the mathematical modelling of the release of dexamethasone from the prepared ulvan membranes. Fitting the power law equation to the obtained data, kinetic parameters that describe the mechanism of release associated to this membrane system were determined. This is a simple semi-empirical mathematical model describing a linear relationship between the logarithm of the amount of drug released and logarithm of time, up to 60% of the maximum drug released (Duarte et al., 2009; Siepmann and Peppas, 2001; Siepmann and Siepmann, 2008).

From the slope of the plot of logarithm of M_t/M_∞ versus logarithm of time, n (the exponent characterizing the release process) was calculated (Fig. 6). In the particular case of ulvan membranes, n is close to 1, which indicates that release of dexamethasone follows a non-Fickian release mechanism, specifically Case-II transport, also described as zero-order kinetic (Siepmann and Peppas, 2001).

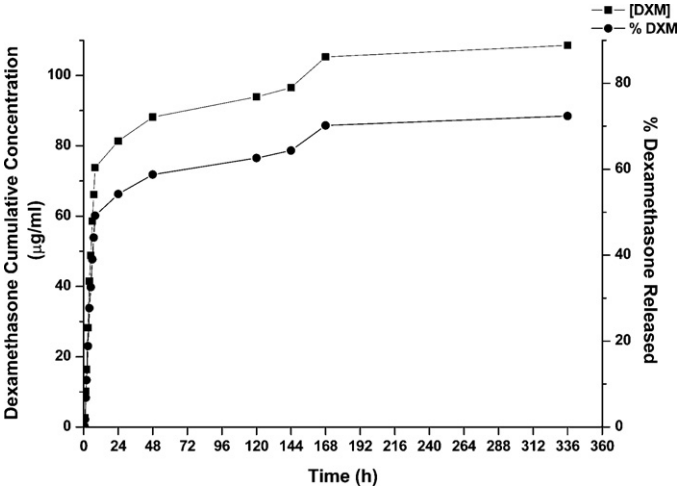


Fig. 4. Dexamethasone concentration and percentage of drug released from ulvan membranes. Results are presented as mean ± SD of *n* samples per time point.

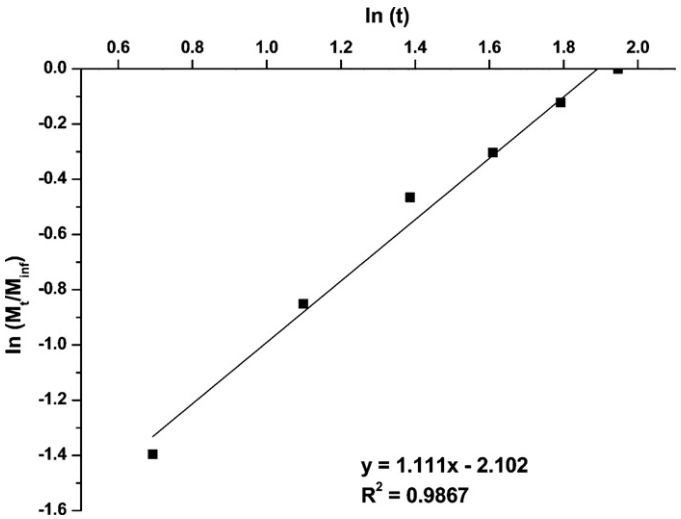


Fig. 6. Power law applied to the drug release profile of ulvan membranes loaded with dexamethasone.

In this particular case, the release of dexamethasone from ulvan membranes is associated with the relaxation of the polysaccharide upon hydration.

4. Conclusions

Ulvan membranes meant to be used as wound dressings or as vehicles to deliver therapeutic agents in the context of wound management have been proposed. As part of the design of these membranes, successful chemical cross-linking of ulvan was achieved with the epoxide BDDE, via the formation of ether bonds through ulvan's hydroxyl groups. The properties of ulvan membranes herein presented suggest that this natural sulphated polysaccharide can be proposed for the envisaged applications as wound dressings. Furthermore, the release of dexamethasone from ulvan membranes proceeds in a sustained fashion, which supports the feasibility of these membranes to be used as drug delivery systems as medicated wound dressings. As the application development of this polysaccharide is in its early stages, the herein described ulvan processing techniques and envisaged applications pose an innovative attempt to add value to this rather unexploited algae derived polysaccharide.

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