Synthesis and Properties of Dendronized Chitosan

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Summary: Chitosan films and microspheres were prepared and their surfaces were functionalized with first generation dendritic molecules. The films were modified by Weisocyanate dendron, while Behera's and bis Behera's amine dendrons were used to modify the microspheres. Prior to dendronization films were prepared by blending chitosan with 18% of polyvinyl pyrrolidone (PVP), and casting the resulting mixture. The degree of dendronization reached was 28%. The microspheres were prepared by coacervation/precipitation, after which the surfaces were activated with either epychlorohydrine (ECH) or 1,4-butanediol diglycidyl ether (BDGE). The oxirane groups were utilized to form covalent bonds between chitosan and dendrons. The degree of dendronization yielded with Behera's amine was 60% for both activating agents. When bis Behera's amine was used, the dendronization reached values of 15 and 21% when ECH or BDGE were used, respectively. The dendronized products were characterized through spectroscopic and microscopic studies and by determination of swelling indexes. Only one of the surfaces was dendronized in every film, which therefore presented a hydrophobic and a hydrophilic surface. Since these films maintain the properties of chitosan, they offer interesting potential as dressings for exuding wounds. The different surfaces make the microspheres potentially applicable as carriers for delivery and controlled release of drugs.

Keywords: biomaterials; dendronization; dendronized chitosan; functional polymers

Introduction

Nowadays, the properties and advantages of dendrimers are well known.[1] Their most defining characteristic is globular shape, as opposed to the random coil structure of linear polymers, which is linked to further interesting effects.[2] Dendrimers have therefore performed a major role in the development of biological applications[3] derived from their multi-branched structure. They constitute perfect scaffolds to present ligands for multivalent interactions, which are found in several biological processes and intercellular interactions such as inflammation, immune response, and infections from bacteria and viruses.[4]

The synthesis of dendrimers is difficult and expensive. Thus, innovative routes to new structures which maintain dendritic effects are being studied, such as the use of dendrons as dendritic or fractal fragments with functional groups on the surface and one different functional group as focal point. Dendronization relates to the covalent or supramolecular interaction of dendrons with non-dendritic substrates in order to create well-defined, stable molecular level nanostructures.

The effects of dendronization are known to be of two types:[5] of a topological nature, according to the architecture and size of dendrons; and of a chemical nature, depending on the chemical structure of the dendrons. Examples, such as the work of Voit et al, illustrate the effects of dendronization on the properties of the materials. They have synthesized a well-defined, linear dendronized diblock copolymer, in which one of the blocks was dendronized

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through a cycloaddition reaction with dendrons of different generation. The phase separation of such dendronized diblock copolymers showed a strong dependence on the generation size.[6] A further example is provided by Haag et al, who have studied the structure-property relationship of methylated and non-methylated polyglycerol dendrons attached to gold surfaces. Their ability to resist the adsorption of proteins from solutions has also been studied.[7]

Chitosan has shown interesting applications as biomaterial [9] due to its excellent biocompatibility, low toxicity, immunostimulatory activity, antibacterial and antifungal action, and anticoagulant properties. Furthermore, degradation products of chitosan have been shown to be non-toxic, non-immunogenic and non-carcinogenic. Hence, our goal is to obtain a new materials by modification of chitosan using dendritic molecules of different generation or chemical structure. The aim of this research is to find new properties in these products and maintain the well-known advantages of chitosan. Thus, different chitosan-based polymers were functionalized with dendritic molecules and the products were characterized showing their properties and potential applications as biomaterials.

Experimental Part

Materials

The following chemicals were purchased and used: 2-hydroxymethyl methacrylate (Aldrich); benzoyl peroxide, BPO (Riedel-de Häen); 1,4 butanediol diglycidyl ether, BDGE (Sigma); epichlorohydrin, ECH (Riedel-de Häen); sodium carbonate (Cicarelli); Behera's amine, dendron 1 (Frontier Scientific); Weisocyanate, dendron 2 (Frontier Scientific); thionyl chloride (Merck); 5-nitroisophtalic acid 99% (Anedra); palladium, 10 wt% on activated carbon, Pd/C (Aldrich); silica gel 60 (Merck); potassium bromide 99% FT-IR grade (Aldrich); chloroform-d 99.8% D (Aldrich); Chitosan, Ch (85% DA, LMW, Aldrich); polyvinyl pyrrolidone, PVP (Todo Droga). Solvents were obtained from Sintorgan, purified by distillation, and dried.

Instruments and Techniques

Fourier Transform Infrared (FT-IR) spectra were obtained on a Nicolet Avatar 360 FT-IR spectrometer on KBr discs. A Thermo Scientific Smart Diffuse Reflectance accessory was used to obtain Diffuse Reflectance Infrared (DRI) spectra. SiC discs were used to abrade the surface of different samples. Attenuated Total Reflectance Fourier Transform Infrared (ATR/FT-IR) interferograms were acquired using the same spectrometer equipped with an Attenuated Total Reflectance accessory. A 45 ZnSe crystal was used to monitor samples. All spectra were obtained with 32 scans at a 4.0 cm^{-1} resolution in a range between 4000 and 650 cm^{-1}.

Nuclear Magnetic Resonance (NMR) spectra were obtained in $CDCl_3$ using a Bruker 400 MHz NMR spectrometer.

Scanning electron micrographs (SEM) of the microspheres were obtained by means of a [Philips SEM 501B] at 12 kV. SEM were performed at the Instituto Nacional de Tecnología Industrial (INTI), Córdoba, Argentina.

The sessile drop technique was used to determine the static contact angles by means of a DataPhysics Optical Contact Angle device. They were carefully measured from the left to the right side of the droplet, and subsequently averaged. These experiments were performed at room temperature.

The equilibrium swelling (E_{sw}) of the films and microspheres was calculated at different pH, according to equation (1),

$$E_{sw}\,(\%) = \frac{W_s - W_o}{W_o} \cdot 100 \qquad (1)$$

where W_s and W_o denote the weight at equilibrium swelling and the initial weight of the film/microspheres, respectively. Each swelling experiment was repeated twice and the average was taken as the E_{sw} value.

Methods

Synthesis of Dendron (3)

5-Nitroisophthalic acid (diacid) was converted into the diacid chloride with thionyl chloride. $SOCl_2$ (10 mL) was added to the diacid (0.4000 g, 16 mmol) in anhydrous THF (10 mL) under nitrogen atmosphere and allowed to react for 4 h. under reflux. The diacid chloride (470 mg, 2.00 mmol) and TEA (0.5 mL) were dissolved in anhydrous THF (50 mL) and reacted with dendron 2 (1.6600 g, 4.00 mmol) for 24 h. under stirring at room temperature. A white solid (3) was obtained and purified by column chromatography (yield 80%). Finally, dendritic molecule 3 (0.8000 g, 0.80 mmol) dissolved in methanol (20 mL) was reduced for 4 h. at 40 psi H_2 at room temperature with 100 mg of Pd/C 10%. Dendron 3 was obtained with a 95% of yield (0.7418 g).[8]

Dendron (3): FT-IR (cm^{-1}): the signals at 1667 and 1535 were assigned to band (C=O stretching vibrations) and band II (N–H bending vibrations) corresponding to the amide group, respectively. The carbonyl absorption band of ester appeared at 1736. ^{13}C NMR ($CDCl_3$) (δ ppm): 173.1 (C=O ester); 163.3 (C=O amide); 148.3 (CNO_2); 136.9 ($C_{3\ and\ 5}$ aromatic); 116.0 (C_4 aromatic); 118.0 ($C_{2\ and\ 6}$ aromatic); 80.9 ($OCCH_3$); 58.4 (CONHC); 30.3 (CH_2CH_2CO); 29.9 (CH_2CH_2CO) and 27.9 ($OCCH_3$). ^1H NMR ($CDCl_3$) (δ ppm): 7.25 (s, 2H, CH aromatic); 7.38 (s, 1H, CH aromatic); 2.25 (m, 12H, CCH_2CH_2CO); 2.08 (m, 12H, CCH_2CH_2CO) and 1.36 (s, 54H, $OC(CH_3)_3$).

Modification of Chitosan

Chitosan was adapted in order to be used in the formation of films and microspheres.

Preparation of Film

The film was prepared from acetic acid solution by the casting method. Chitosan powder (1.0000 g) was dissolved in 1.5% acetic acid solution (100 mL). PVP powder (0.1800 g) was added to the chitosan solution. Then, this solution (50 mL) was cast on a glass plate (10 cm of diameter), gradually dried in air at room temperature.

Dendronization of Films

A 25 mL nitrogen flask equipped with a magnetic stirring bar was charged with a chitosan-PVP film (0.100 g), 10 mL dimethylacetamide, dendron 1 (0.150 g, 0.15 mmol), and dibutyltin dilaurate (0.02 mL) were added and stirred at 60 °C for 4 days. The film was washed with $CHCl_3$ to remove unreacted dendron, and the Chitosan-PVP-dendron 1 film was dried under vacuum. The 8.67×10^{-5} mol dendron by 1.000 g of the film were determined by the pyridinium chloride method.[10]

Preparation of Microspheres

Chitosan microspheres were prepared by dissolution of polymer (1.000 g) in a 5% (V/V) acetic acid solution (35 mL). Chitosan solution was then added dropwise by a dropper into a precipitation bath containing 0.5 M NaOH solution (500 mL). This step neutralized the acetic acid within the chitosan gel, thus coagulating the gel to spherical uniform chitosan gel beads. A magnetic stirrer was used to stir the aqueous NaOH solution continuously at 200 rpm to prevent chitosan beads from sticking to one another or to the glassware surface. The wet chitosan beads formed from neutralization of the excess acid on the surface of the chitosan gel were filtered and extensively rinsed with distilled water to remove NaOH; then, they were air-dried to eliminate water. After that, the microspheres were modified with ECH and BDGE according to the following procedure:

Ch-ECH (M_I) and Ch-BDGE (M_{II}) microspheres: 0.500 g of chitosan microspheres were added to an 0.1 M spacer (ECH or BDGE) solution (30 mL) to obtain a 1:1 ratio with chitosan (mol spacer: mol CH_2OH). The chitosan beads in spacer solution were heated to 45 °C for 2 h. and stirred continuously using a magnetic stirrer at 200 rpm. Afterwards the beads were filtered, washed with distilled water to remove excess ECH or

BDGE, and air-dried. The oxirane groups were determined by the pyridinium chloride method.[10]

Dendronization of Ch-ECH (M_I) and Ch-BDGE (M_{II}) Microspheres

The epoxy-activated matrices, Ch-ECH (M_I) and Ch-BDGE (M_{II}) microspheres, were swollen in water (100 mg mL^{-1}) for 24 h. Dendrons 2 or 3 (4.50 mg and 10.7 mg, respectively), dissolved in 0.2 M Na_2CO_3 (0.08 mL), were then added to the microspheres and stirred at 60 °C for 36 h. The mol ratio used was 1:0.6 corresponding to the epoxy:dendron 2 or 3, respectively. Products Ch-ECH-dendron 2 ($M_{I.2}$), Ch-ECH-dendron 3 ($M_{I.3}$), Ch-BDGE-dendron 2 ($M_{II.2}$), and Ch-BDGE-dendron 3 ($M_{II.3}$) were filtered and washed with water, 0.1 N acetic acid solution and water again. The products were dried.

The incorporation of the dendrons was estimated by titration of epoxy groups by the pyridinium chloride method.[10]

Results

Synthesis of Dendrons

The dendrons used for the modification of chitosan were Weisocyanate (dendron 1), Behera's amine (dendron 2) and bis Behera's amine (dendron 3) (Figure 1). They have tert-butyl functional groups on the surface, and amine or isocyanate groups as a focal point, which will be used for the covalent bond with chitosan. Dendrons 1 and 2 were acquired commercially, while dendron 3 was synthesized in our laboratory, which has already been reported.[8]

Dendron 1 was selected to modify the films because urethane and urea groups formed on the surface are important in order to deal with specific surface properties in future applications.

Dendrons 2 and 3 were chosen for dendronization of the microspheres because their amine focal points can act as nucleophiles to open the oxirane ring. It has been demonstrated that these dendrons are biocompatible and suitable for biological applications.[14]

Modification of Chitosan

Chitosan was adapted in the formation of film and microspheres for subsequent surface dendronization (Figure 2). Preparation of films and microspheres (M) was optimized as follows:

Preparation of Film

Since chitosan has non-toxicity, biocompatibility, high hydrophilicity, good complexation and film-forming ability as its properties, it has found a wide range of biomedical applications.[11] It was mixed with PVP by blending, which is one of the most effective methods for providing new materials suitable for use in the biomedical field. Marsano et al. and Sakurai et al [12,13] have extensively studied Chitosan-PVP blends and concluded that these blends are

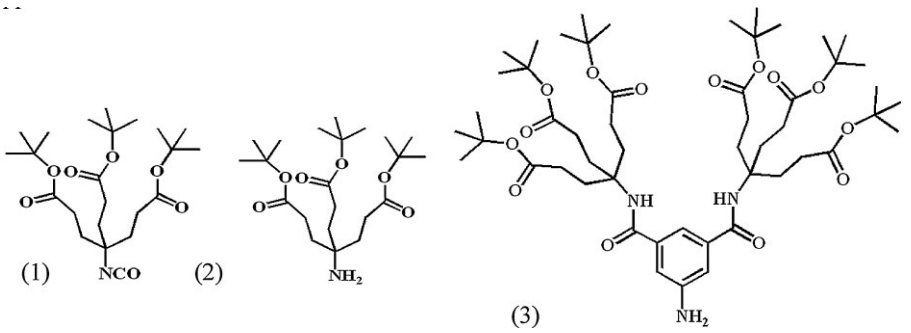

Figure 1.
First-generation dendritic molecules: weisocyanate (1), Behera's amine (2) and bis Behera's amine(3).

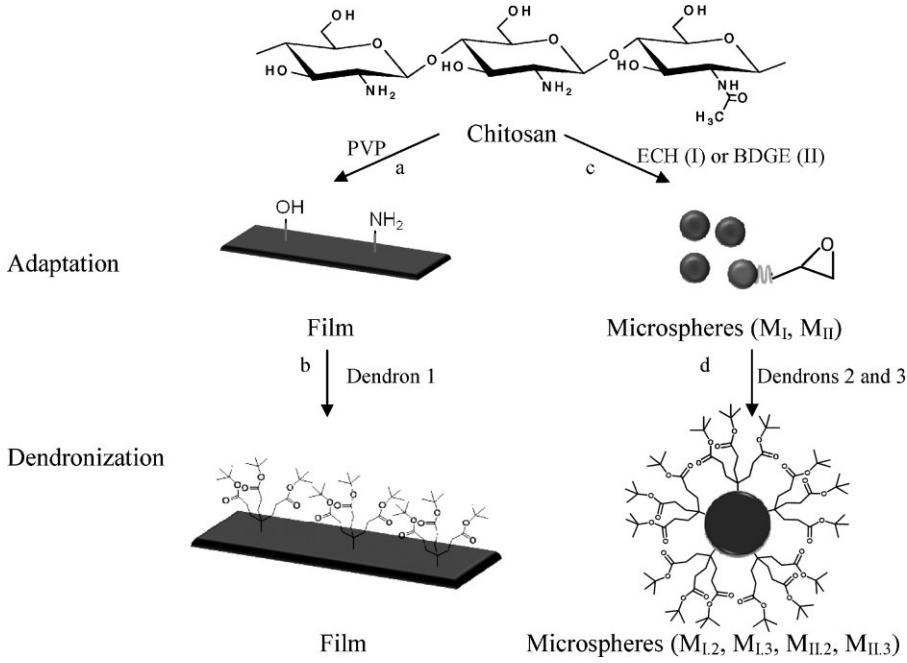

Figure 2.
Synthesis of films and microspheres ($M_{I.2}$, $M_{I.3}$, $M_{II.2}$, $M_{II.3}$) of dendronized chitosan.

miscible in the solid state and interact at molecular level. Chitosan and PVP were mixed and the film was made by casting. The ATR/FT-IR spectrum of the film showed the PVP band at 1670 cm^{-1} corresponding to carboxyl groups. In addition, we observed other characteristic peaks of chitosan at 1590 and 1567 cm^{-1} corresponding to the N–H bending and to the H–N–H clipping vibration of amine groups, respectively. The broad and strong band ranging from 3200 to 3600 cm^{-1} indicates the presence of –OH and $-NH_2$ groups, which is consistent with the peak at 1104 and 1150 cm^{-1} assigned to alcoholic C–O and C–N stretching vibration, respectively. The peaks at 2945 and 2840 cm^{-1} can be assigned to asymmetric and symmetric $-CH_2$ groups.

Dendronization of Film. The film was dendronized by Weisocyanate dendron (1). Ch-PVP-dendron film was obtained with a degree of dendronization of 28% (difference in weight before and after dendronization). ATR/FT-IR analysis of the sample shows that the expected product was obtained, and also that dendronization occurred only on one surface of the film. On this face, the characteristic peaks of the dendron, as the tert-butyl methyl group at 850 and 756 cm^{-1}, 2968 cm^{-1} assigned to the CH_3 stretching and the band at 1225 cm^{-1} corresponding to C–O–C of the ester group were observed. The band at 1729 cm^{-1} can be assigned to the C=O stretching of the dendron or urethane or urea bond formation, because the vibrations overlap. The characteristic bands of chitosan-PVP showed no changes in the profile of the spectrum of the other side of the film.

Studies of the properties of the film. Swelling index. After the dendronization process, the equilibrium swelling (E_{sw}) of the film was studied at different pH: 3.2 -which is the pH of exuding wounds- and 7.4. E_{sw} values were determined according to equation (1).

The water contact angles were 111.5 ± 0.7 and 82 ± 1 for the Chitosan-PVP-dendron (dendronized surface), and for Ch-PVP (non dendronized surface), respectively.

Preparation of Ch-ECH (M_I) and Ch-BDGE (M_{II}) Microspheres

Microspheres of chitosan were modified through reaction with epyclorhydrine (ECH, I) and 1,4-butanediol diglycidyl ether (BDGE, II). They were used as spacer arms and crosslinking agents to facilitate the subsequent covalent bond of the dendrons and to improve the stability of the microspheres. The spacer-chitosan reaction could also affect the hydroxyl groups but it is known that amine groups are better nucleophiles than hydroxyl groups.

In both cases, the surfaces of the microspheres were activated with epoxy groups. Modification of the surfaces of the microspheres was clearly shown by diffuse reflectance IR. The spectrum of M_I revealed a new signal at $3027\,cm^{-1}$ corresponding to C–H stretching vibration of the epoxy group, the stretching vibration of C–O–C appears at 1033, 1249 and $827\,cm^{-1}$.

The C–H stretching corresponding to the epoxy group of the BDGE in spectrum of M_{II} appears at $3035\,cm^{-1}$ and at 1240 and $824\,cm^{-1}$ the bands assigned to the asymmetry and symmetry stretching vibration C–O–C of the ether group, respectively. In both cases also the typical absorptions of chitosan were present as previously described.

Dendronization of Microspheres M_I and M_{II}. The microspheres (M_I and M_{II}) were dendronized using Behera's amine (2) and bis Behera's amine dendrons (3), obtaining Chitosan-ECH-dendron 2 ($M_{I.2}$), Chitosan-ECH-dendron 3 ($M_{I.3}$), Chitosan-BDGE-dendron 2 ($M_{II.2}$), Chitosan-BDGE-dendron 3 ($M_{II.3}$). The dendronized spheres were characterized by Diffuse Reflectance-IR and all expected products were obtained. The typical band at $1729\,cm^{-1}$ corresponding to C=O stretching of the ester dendron confirms the presence of this. Other changes in the spectra were not observed for the dendronized sample due to the high intensity of the absorption bands of Chitosan. Table 2 shows the percentage incorporation of the dendrons.

Table 1.
Swelling studies at 3.2 and 7.4 pH of film.

Matrix	E_{sw} (%)	
	pH 3.2	pH 7.4
Ch-PVP	561	137
Ch-PVP-W	1262	113

Table 2.
Extent of conversion of functional groups and amount of bound dendritic molecules for microspheres.

Matrix	Epoxide groups (mol/g matrix)	Dendron (mol/g matrix)	Yield of reaction: bind of dendron (%)
$M_{I.2}$	1.80×10^{-4}	1.08×10^{-4}	60
$M_{I.3}$	1.80×10^{-4}	2.70×10^{-5}	15
$M_{II.2}$	3.78×10^{-4}	2.27×10^{-4}	60
$M_{II.3}$	3.78×10^{-4}	8.00×10^{-5}	21

Studies of the Properties of Microspheres $M_{I.2}$, $M_{I.3}$ $M_{II.2}$ and $M_{II.3}$. The dendronized chitosan microspheres were characterized by SEM and swelling studies. The mean diameter was in the range of 0.8–1.2 mm. The spheres were homogeneous with no variations in size after dendronization (Figure 3).

Swelling at pH 1.2 and 7.4 was studied for different microspheres and the E_{sw} values were determined according to equation (1). pHs 1.2 and 7.4 have been selected because they represent stomach and intestinal conditions, respectively (Figure 4).

Discussion

The films were obtained with a degree of dendronization of 28%. The dendronization was observed only on the film's surface which was in contact with the dendron solution.

Swelling was higher at minor pH due to the positive charges of the amino groups of chitosan and the electrostatic repulsion of

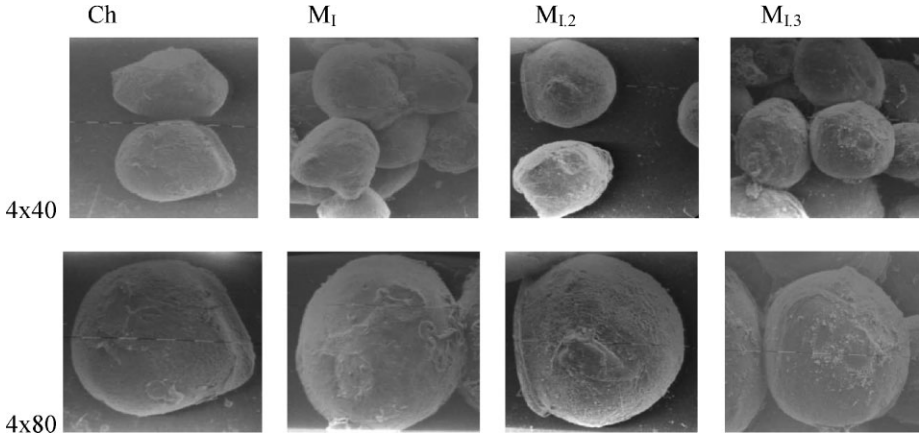

Figure 3.
Scanning electron micrograph for chitosan microspheres, M_I, $M_{I.2}$ and $M_{I.3}$.

their chains, while at pH 7.4 no significant changes in swelling were observed. Dendronization of chitosan increased the swelling in relation to the starting chitosan.

Contact angle studies proved the hydrophobicity of one of the surfaces of the films, which had previously been modified by dendronization. The presence of t-butyl groups of the dendron gives hydrophobicity to one surface of the film. However, the swelling capacity of the film as a whole increased, probably due to the presence of the dendron, which disrupted the interaction between the chitosan chains near the surface and allowed an easier entry of water molecules.

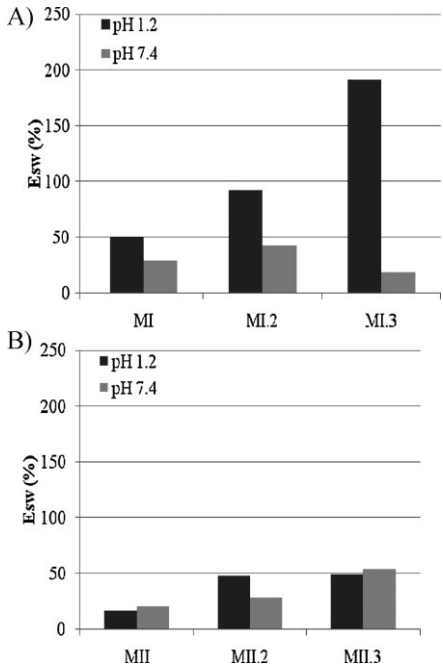

Figure 4.
Swelling index (E_{sw}) of microspheres. (A) M_I, $M_{I.2}$ and $M_{I.3}$; (B) M_{II}, $M_{II.2}$ and $M_{II.3}$.

In the microspheres ($M_{I.2}$, $M_{I.3}$, $M_{II.2}$ and $M_{II.3}$) the amount of dendron 2 incorporated onto the structures was higher than that of dendron 3, probably due to steric hindrance. In addition, the longer spacer (BDGE) allowed a higher incorporation of dendron 2 and 3 than ECH.

When ECH was used, the swelling of the dendronized products at pH 1.2 was higher. At the lowest pH value tested (1.2), where chitosan was positively charged, swelling was high owing to the repulsion between their chains. Dendronized products M_{I3}, showed higher swelling than M_{I2}. Thus, an increase in the size of the dendron produced a marked increase in swelling (Figure 5A) owing to a disruption of the interactions between the chains. At the highest pH examined (7.4) M_{I2} and M_{I3} showed no important difference in relation to non-dendronized microspheres. When BDGE was used the swelling of the products was minor with respect to ECH (Figure 5B). In addition, swelling was

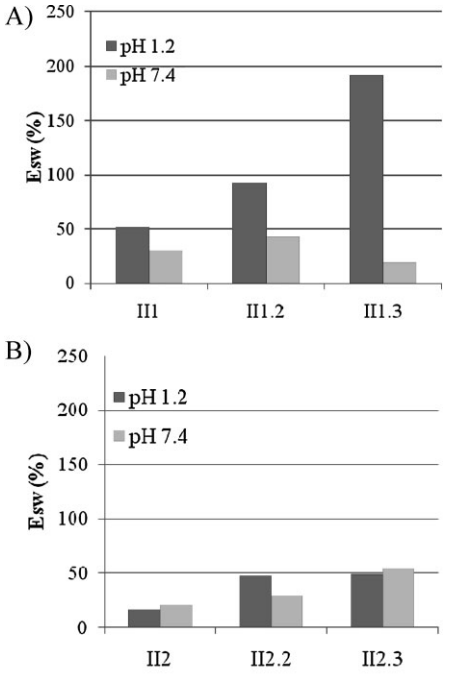

Figure 5.
Swelling index (Esw). (A) Ch-ECH (II_1, $II_{1\text{-}2}$ and $II_{1\text{-}3}$). (B) Ch-BDGE (II_2, $II_{2\text{-}2}$ and $II_{2\text{-}3}$) microspheres.

practically unaffected by either pH or size of dendrons.

Probably when ECH was used to modify the spheres of chitosan, dendronization occurred only on the surface, while with BDGE, a penetration of the dendrons into the sphere could be possible. The better reactivity and affinity of BDGE to chitosan with respect to ECH produced a higher cross-linking inside and outside of the spheres, giving less swelling at both pH. In this case, higher percentages of dendron incorporation were also found. ECH could therefore be linking the dendrons only to the surface of the spheres, while BDGE could be the best cross-linker and linker of the dendrons, inside and outside the microspheres.

Conclusion

The dendronized films prepared with chitosan showed the following major properties: one hydrophilic and one hydrophobic surface, good absorbing capacity in acid medium, and the presence of urethane and urea groups in the film/dendrons interface. In addition, the main advantages of chitosan include biocompatibility, antimicrobials, mucoadhesion, and softness when applied on the skin. It is known that dressings for heavily exuding wounds must have specific properties, namely germ-destructive (bactericidal), gel-forming in contact with wound fluid, and soft for a protective cushioning effect on the wound. It is recommendable to have a hydrophobic non-woven outer surface for reducing the tendency for any dressing to adhere to the wound, and a hydrophilic core with a high capillary activity through which exudates can pass quickly, thus preventing wetness accumulation and reducing the risks of maceration and infection.[15] The dendronized film obtained in this research work showed these properties, thus making this material potentially useful as biomaterial, more specifically in the making of dressings for exuding wounds.

In relation to the dendronized spheres prepared with the well-known advantages of chitosan, the possibility of providing their surface with multivalent properties through the introduction of specific molecules (such as solubilizing molecules, or specific pharmaceutical drugs) makes this material potentially applicable to different therapies to be used as macrocarriers for the controlled release of drugs.

[1] F. Vögtle, G. Richardt, N. Werner, "*Dendrimer Chemistry. Concepts, Synthesis, Properties, Applications*", Wiley VCH Publisher, **2009**.
[2] G. R. Newkome, C. N. Moorfield, F. Vögtle, "*Dendritic Molecules. Concepts, Synthesis, Perspectives*", VCH Publisher, Germany 1996, chap 1 and chap 2.
[3] W. D. Jang, K. M. Kamruzzaman Selim, C. W. Lee, I. K. Kang, *Prog. Polym. Sci.* **2009**, *34*, 1.
[4] S. Galeazzi, T. M. Hermans, M. Paolino, M. Anzini, L. Mennuni, A. Giordani, G. Caselli, F. Makovec, E. W. Meijer, S. Vomero, A. Cappelli, *Biomacrom.* **2010**, *11*, 182.
[5] H. Frauenrath, *Prog. Polym.* **2005**, *30*, 325.
[6] S. Fleischmann, A. Kiriy, V. Bocharova, C. Tock, H. Komber, B. Voit, *Macrom. Rapid Commun.* **2009**, *30*, 1457.

[7] M. Wyszogrodzka, R. Haag, *Langmuir.* **2006**, *25*(10), 5703.
[8] M. Martinelli, M. Calderón, C. Alvarez, I. M. Strumia, *React & Funct Polym.* **2007**, *67*, 1018.
[9] H. Sashiwa, S. Aiba, *Prog. Polym. Sci.* **2004**, *29*, 887.
[10] H. Lee, K. Neville, ''*Handbook of Epoxy Resins*'', McGraw-Hill Inc, New York 1967, p. 17.
[11] M. Ignatova, N. Manolova, I. Rashkov, *Eur. Polym. J.* **2007**, *43*, 1112.
[12] E. Marsano, S. Vinici, J. Skopinske, M. Wisiniewski, A. Sionkowska, *Macrom. Symp* **2004**, *218*, 251.
[13] K. Sakurai, T. Maegawa, T. Takahashi, *Polym.* **2000**, *41*, 7051.
[14] L. Fernandez, M. Calderón, M. Martinelli, M. Strumia, H. Cerecetto, M. González, J. J. Silber, M. Santo, *J. of Phys. Org. Chem.* **2008**, *21*, 1079.
[15] http://en.hartmann.info/images/New_Zetuvit_Plus.pdf.

