ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Wound dressing membranes based on chitosan: Preparation, characterization and biomedical evaluation

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Manuscript Info

Manuscript History:

Received: 18 June 2015
Final Accepted: 29 July 2015
Published Online: August 2015

Key words:

Chitosan, membranes, Schiff bases, Cinnamaldehyde, Wound dressing

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Abstract

Chitosan based film for biomedical applications were prepared by casting and drying in presence of different amount of cinnamaldehyde. Chemical and physical studies were done for prepared membranes. Ion exchange capacity of membranes was used for monitoring coupling process between carbonyl of cinnamaldehyde and amine of chitosan. Results shown a dramatic decreased (from 5.11 to 2.73 meq/g) by increasing amount of cinnamaldehyde as a result of decreasing of amino groups along polymer backbone. Some physical characterization of membranes were measured and recorded which exhibited change in the polymer nature from hydrophilic to hydrophobic nature such as water uptake, moisture content and wettability. Electronic spectrum and color measurements show change in the membranes color from transparent to yellow with decreasing in the membranes transmission. Thermal analysis of prepared membranes (TGA and DSC) confirms the modification and show change of thermal stability of modified membranes. Mechanical analysis of membranes show breakdown of its mechanical properties due to disappearance of hydrogen bond of amine groups. Bio-evaluation of the fabricated membranes to be used as wound dressing membranes was investigated. In addition; Measuring of haemocomptability, thrombogenicity, biodegradability, blood clotting index and protein adsorption of such membranes were studied.

INTRODUCTION

The healing of a skin wound is complicated courses, including a wide range of cellular, molecular, physiological, and biological processes. Immediate coverage using wound dressing is a cornerstone of wound management. The wound repairs in cases of acute, chronic, more extensive wounds, or skin loss of the oldest would be inevitable unless some skin substitutes are used [**Metcalfe and Ferguson., 2007].** The following characteristics are required for ideal wound and burn dressing • ease of application • bioadhesiveness to the wound surface • sufficient water vapor permeability • easily sterilized • inhibition of bacterial invasion • elasticity and high mechanical strength • compatibility with topical therapeutic agents • optimum oxygen permeability • biodegradability • non-toxic and non-antigenic properties. **[Sheridan and Tompkins., 1999; Balasubramani et al., 2001; Jones et al., 2002]**
Chitosan has novel properties such as biocompatibility, biodegradability, antibacterial, haemostatic properties and wound-healing activity. Chitosan is known in the wound management field for its haemostatic properties. Further, it also possesses other biological activities and affects macrophage function that helps in faster wound healing. The

biological properties including bacteriostatic and fungi static properties are particularly useful for wound treatment. **[Moore and Roberts., 1981; Muzzarelli et al; 1985].** Increase of antimicrobial activity of chitosan take attention of scientists during last years to face continuous mutation of bacteria. In this area several novel derivatives were prepared including amination **[Mohy eldin et al., 2008a; 2012; 2013],**alkylation and arylation **[Rabea et al., 2004;2005;2006]**, schiff base formation **[Mohy eldin et al., 2015; Kenawy et al., 2015; Soliman et al., 2013]** .etc.
The object of this work is to promote antimicrobial activity of chitosan membranes using cinnamaldehyde (the mean component of cinnamon essential oil). Studies of physical and chemical behavior of modified membranes were done beside biomedical evaluations.

2. Materials and methods

2.1 Materials

Chitosan prepared in our laboratory from localized shrimp skeletons. Shrimp skeletons provided from commercial resource, Sodium hydroxide pellets (Purity 99-100 %, M.W.40). Cinnamaldehyde (Purity 98 %, M.W.132, Scharlau, Spain). Acetic acid (Purity 99.8%, M.W.60.05). Ethanol (Purity 99.9%, M.W.46.07). International co for Supp&Med. Industries, (Egypt). Monosodium phosphate (Purity 98 %, M.W.119.98, Sigma, Germany). Disodium phosphate (Purity 98 %, M.W.117.98, Sigma, Germany). Sodium chloride (Purity 99.5%, M.W.58.44).

2.2 Methods

2.2.1. Membrane preparation and characterization

2.2.1.1. Preparation of membranes

Membrane was prepared as follows: 1 g of chitosan (Ch) was dissolved in 50 ml 2% acetic acid. Different amount of Cinnamaldehyde (Cin) was added dissolved in ethanol (from 0.1 to 1.5 ml), solution was stirred well for 2 hour in room temperature, and then strained through cheesecloth to remove any undissolved particles. On a clean Petri dish, the solution was casted at room temperature for 48 hours to ensure complete solvent evaporation. Once the membrane was dried and separated from the Petri dish it was rinsed with 50 ml of 1 M of NaOH. Rinsing of the membrane in a caustic solution gives the film water-resistance by neutralizing and removing any acetic acid residue present in the membrane. The membrane was then washed with distilled water to remove any traces of alkali and to neutralize it. Finally, the wet membranes were spread out and attached to the clean glass support with clamps and allowed to dry for 24 hours at room temperature.
Seven different molar ratios of chitosan and cinnamaldehyde were studied and coded as sample Ch/Cin 0.1, Ch/Cin 0.25, Ch/Cin 0.5, Ch/Cin 0.75, Ch/Cin 1.0, Ch/Cin 1.25 and Ch/Cin 1.5 in addition to native chitosan membrane (Ch/Cin 0)

2.2.2. Structure verification

2.2.2.1. UV-Vis Spectroscopic analysis

The electronic absorbance of chitosan and chitosan derivative membranes were done using spectrophotometer scanned from 190 -1000 nm.

2.2.2.2. Thermal gravimetric analysis (TGA)

TGA Analysis of chitosan and chitosan derivative membranes were carried out using Thermal gravimetric Analyzer (Shimadzu TGA –50, Japan).

2.2.2.3. Scanning electron microscope (SEM)

Scanning of chitosan and chitosan derivative membranes were carried out using Scanning Electron Microscope (Joel Jsm 6360LA, Japan).

2.2.3. Physico-chemical properties

2.2.3.1. Determination of ion exchange capacity of chitosan membranes.

Chitosan is insoluble in sulfuric acid. A known weight of chitosan or chitosan derivative membranes were added to known volume of 0.1 M H_2SO_4 solution and the mixture was kept in shaking for 2 h. The mixture was filtered and an aliquot was titrated against standard solution of sodium hydroxide. Similarly control titration without the addition of chitosan was also run. From the difference in the volume of NaOH required for neutralization, ionic capacity of chitosan samples were calculated using following equation (2.1):

$$\text{Ion exchange capacity} = (V_2 - V_1)\, a / w \quad \text{(meq/g)} \qquad \text{eq. (2.1)}$$

Where $\mathbf{V_2}$ and $\mathbf{V_1}$ are the volumes of NaOH required for complete neutralization of H_2SO_4 in the absence and presence of chitosan membrane, respectively, **a** is the normality of NaOH and **w** is the weight of sample taken for analysis **[Ramnani and Sabharwal., 2006]**.

2.2.3.2. Mechanical properties

Mechanical properties were done to characterize the chitosan membranes and to confirm reproducibility of the membrane formation technique. These properties include the membrane thickness, and maximum stress and strain to failure. A method for testing the tensile properties of the film was adopted according to ASTM D- 882 standards for testing tensile properties of paper and paper broad using a constant-rate of elongation apparatus. The instrument that used to test these properties was an AG-1S, SHIMADZU. Membrane thickness measurements were obtained with an electronic digital micrometer.

2.2.3.3. Surface roughness

The surface roughness of the substrate used for wound dressing is very important. The average roughness was measured using surface roughness tester SJ- 201P, Japan. Samples were mounted onto a glass slide with double-sided tap. Minimum sample dimensions were 25mm X 25mm. All results are the average of triplicate measurements. **[Mohy eldin et al., 2008b]**

2.2.3.4. Water uptake

Water uptake (%) estimation was performed by placing a weighed sample previously dried in distilled water. After 6 hours to reach to equilibrium swelling, the sample was then filtered off, carefully bolted with a filter paper and weighed. The water uptake, W, calculated by applying the following equation **(2.1);**

$$\textbf{Water uptake \% = [M – Mo] / Mo X 100} \qquad \textbf{eq. (2.1)}$$

Where **M** is the weight of the swelled sample and $\mathbf{M_0}$ is the weight of the dry sample.

2.2.3.5. Moisture content

Films placed in humidity champers with humidity ratio 80% overnight and then weighed before and after drying in an oven at 105 ^{o}C for 3 h. Water content was calculated as follows equation **(2.2).**

$$\textbf{Moisture content \% = [M – Mo] / Mo X 100} \qquad \textbf{eq. (2.2)}$$

Where **M** is the weight of the sample before drying and $\mathbf{M_0}$ is the weight of the dry sample.

2.2.3.6. Optical properties

Film color was measured by an X-Rite Model Sp64 Made n USA. The colorimeter was calibrated with white and black plates. A white standard color plate for the instrument calibration was used as a background for color measurements of the films. The system provides the values of three color components; L* (black-white component, luminosity), and the chromaticness coordinates, **a*** (+red to −green component) and **b*** (+yellow to −blue component). Color differences Δ**E*** were also calculated by the following equation **2.3**:

$$\Delta \boldsymbol{E} = \sqrt{(\Delta \boldsymbol{L}^*)^2 + (\Delta \boldsymbol{a}^*)^2 + (\Delta \boldsymbol{b}^*)^2} \qquad \textbf{eq. (2.3)}$$

Where: $\Delta L^* = L^* - L_0^*$, $\Delta a^* = a^* - a_0^*$, $\Delta b^* = b^* - b_0^*$

Being**:** $\mathbf{L^*_0}$; $\mathbf{a^*_0}$; $\mathbf{b^*_0}$ are the color parameter values of the standard and **L***; **a***; **b*** the color parameter values of the sample.

2.2.3.7. Contact angle measurements

Static water contact angle measurements were performed at room temperature using (advanced Gonimeter model 500-F1) in a sessile drop configuration (using ultrapure water as the liquid), coupled with a video camera and image analysis software. At least ten droplet images were obtained for each film.

2.2.4. Membrane bio-evaluation

2.2.4.1. Evaluation of haemocomptability

The haemolysis tests were performed as described in American Society for Testing and Materials (ASTM) (ASTM F 756-00, 2000). Anti-coagulated blood was used for this purpose. This sample was prepared by adding 1 ml of anticoagulant acid citrate dextrose solution (ACD) **[US Pharmacopeia XXIII., 1994**] to 9 ml of fresh blood. Before performing the tests, Samples (1 cm^2) were placed in polypropylene test tubes and 7ml of phosphate buffer solution pH= 7.0 (PBS) were added. After 72 hrs of incubation at 37℃, the PBS was removed and 1ml ACD blood (9.02 mg/ml) was added to each sample and maintained at 37℃ for 3 hrs. Positive and negative controls were prepared by adding the same amount of ACD blood to 7ml of water and PBS, respectively. Each tube was gently inverted twice each 30 min to maintain contact of the blood with the material. After incubation, each fluid was transferred to a suitable tube and centrifuged at 2000 rpm for 15 min. The hemoglobin released by haemolysis was measured by the optical densities (OD) of the supernatants at 540 nm using a spectrophotometer (Model Ultrospec 2000). The percentage of haemolysis was calculated as follows equation 2.4:

$$\textbf{Haemolysis (\%)} = [(OD_{\text{sample}} - OD_{\text{negative control}}) / (OD_{\text{positive control}} - OD_{\text{negative control}})] \qquad \textbf{eq. (2.4)}$$

According to ASTM F 756-00 (2000) materials can be classified in three different categories according to their haemolytic index (haemolysis %):materials with percentages of haemolysis over 5% are considered haemolytic; while the ones with haemolytic index between 5% and 2% are classified as slightly haemolytic. Finally, when the material presents a haemolysis percentage below 2% it is considered as a non-haemolytic material.

2.2.4.2. Thrombogenicity

Evaluation of thrombus formation on polymeric surfaces was carried out using a gravimetric method **[Imai and Nose., 1972].** Anticoagulated rabbit blood was used for this purpose. This sample was prepared by adding 1mL of anticoagulant acid citrate dextrose solution (ACD) **[USP Pharmacopeia XXIII., 1994]** to 9mL of fresh rabbit blood. Before performing the tests, membranes were immersed in PBS at a constant temperature of 37℃. After 48 h of incubation, the PBS was removed and the ACD blood was put in contact with the surface of the polymers and also to an empty Petri dish, which acted as a positive control. Blood clotting tests were initiated by adding 0.02mL of a 10M calcium chloride solution and were stopped after 45 min by the addition of 5mL of water. Resultant clots were fixed with 5mL of a 36% formaldehyde solution and were then dried with tissue paper and finally weighed.

2.2.4.3. Dynamic blood clotting test

According to **Zhou and Lin (1997),** dynamic blood clotting was measured. A series of membranes were placed at the bottoms of 100 ml beakers. Beakers were placed in a thermostat at 37^{o}C for 5min and 0.25 ml ACD whole blood was dropped on the surface of these membranes, followed by the addition of 0.02 ml $CaCl_2$ solution (0.2 mol/l) to the blood sample. The beakers containing blood sample were kept in a thermostat at 37^{o}C. The blood clotting test was carried out by spectrophotometric measurement of the relative absorbency of blood sample that had been diluted by 50 ml distilled water at 540 nm. The absorbency of solution of 50 ml distilled water and 0.25 ml ACD whole blood at 540nm was assumed to be 100, which was used as reference value. That is to say, the blood clotting index (BCI) of biomaterial can be quantized by the following equation 2.5:

$$BCI = (A / Ao) \times 100 \qquad \text{eq. (2.5)}$$

Where **A** and $\mathbf{A_o}$ are Absorbance of blood contacted with sample and solution of distilled water and ACD blood respectively

2.2.4.4. Protein adsorption

Adsorption experiments were made with BSA. Standard curve was done using concentration between 0.05 g/dl and 0.5 g/dl in phosphate buffered saline (PBS, pH 7.4). The membranes with an area of 1cm^2 (each piece) were incubated in PBS for 24 h, and then immersed in the certain protein solution for 3 h. After protein adsorption, membranes were carefully removed and protein adsorbed was estimated by measuring protein concentration before and after immersing membranes at wavelength 280nm**. [Ishihara et al., 1999].**

2.2.4.5. Evaluation of biodegradability

Degradation activities of membranes were determined by colorimetric method of **Miller (1959)** using the dinitrosalicylic acid DNS-reagent. This method is based on determination of color developed after reaction between reducing sugars liberated from polysaccharide and DNS-reagent

Procedure: Two ml of phosphate buffer (pH 7.0) including membranes were transferred to 2 ml Eppendorf. 0.5 ml of lysozyme solution was added to it and incubated four 24 hr at 37^{o}C. Blanks were prepared in the same way without enzyme. The determination was carried out in triplicate. After incubation, enzyme activity was stopped by adding 1.5 ml DNS-reagent; tubes were placed in a boiling water-bath for 15 min, cooled down to room temperature. optical density (OD) of the samples was immediately measured at 575 nm.

3. Results and discussion

According to the results obtained in our previous work, antimicrobial activity of Cinnamyl chitosan schiff base increase by increase degree of substitution, in the other hand solubility of formed schiff bases were decreased. **[Mohy Eldin et al., 2015].** In our work, highly substituted cinnamyl chitosan membranes were prepared, reaction between chitosan and cinnamaldehyde was done during casting process in Petri dishes to have transparent yellowish membranes (Figure 1). The prepared membranes were characterized by different analyses related to their chemical structure, physicochemical properties, thermal properties and finally, their bio-applications as wound dressing membranes. Wider range of chitosan-cinnamaldehyde ratios were tested in this part.

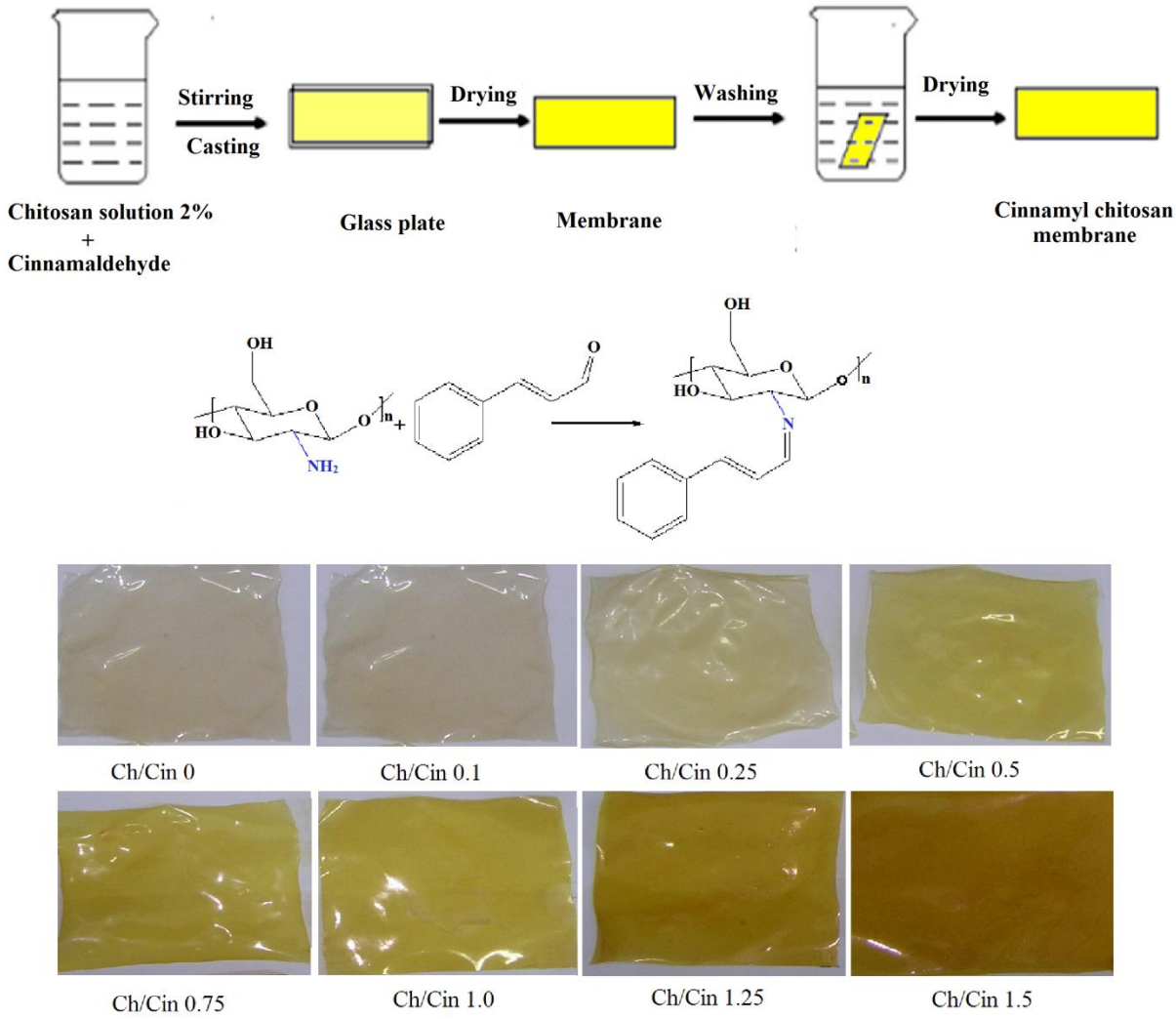

Figure 1: schematically preparation of Cinnamly chitosan schiff base membranes

3.1. Physicochemical analyses

3.1.1 Ion exchange capacity

Ion exchange capacity of chitosan membranes was measured (Figure 2). It was shown that a dramatic decrease in ion exchange capacity was attributed to consumption of surface free amine groups on coupling with cinnamaldehyde.

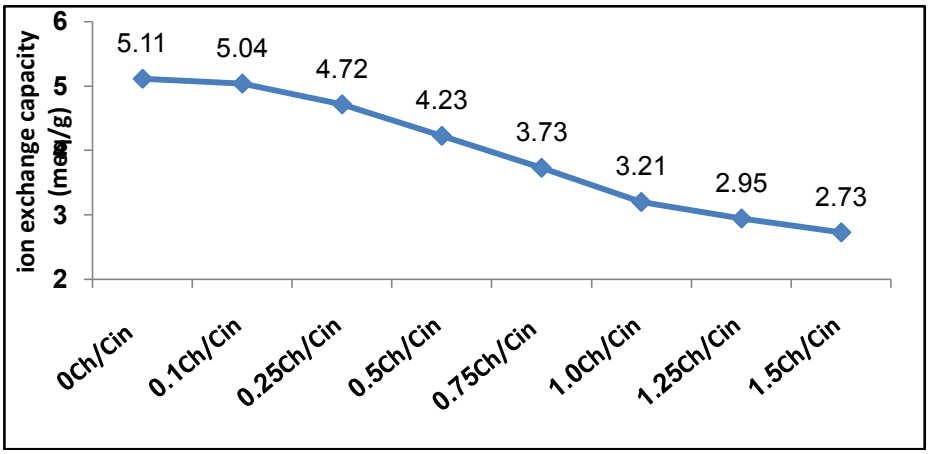

Figure 2: ion exchange capacity of chitosan membranes with different content of Cinnamaldehyde

Depression of ion exchange capacity proves the chemical bond built between cinnamaldehyde and chitosan.

3.1.2 Water up take

Water up take of chitosan and chitosan Schiff bases were determined and illustrated in Figure 3. Water sorption of chitosan membranes was attributed to its hydrophilic groups (i.e.; hydroxyl and amine groups). Figure shows three stages of gradual decrease of membrane water uptake by increase cinnamaldehyde concentration; slightly decrease up to 0.5 moles, beyond these concentrations a dramatic decrease was observed till 1.0 mole, then with a lower rate up to 1.5 moles. Changing of hydrophilic/hydrophobic nature of membranes by modification may be explaining this phenomenon. Substitution of NH_2 hydrophilic groups with hydrophobic cinnamaldehyde nucleus is responsible for such decrease in water uptake in general. At certain degree of substitutions, the cinnamaldehyde nucleus blocked the inter chain space filled with in piped water molecules which cause a dramatic decrease of water uptake.

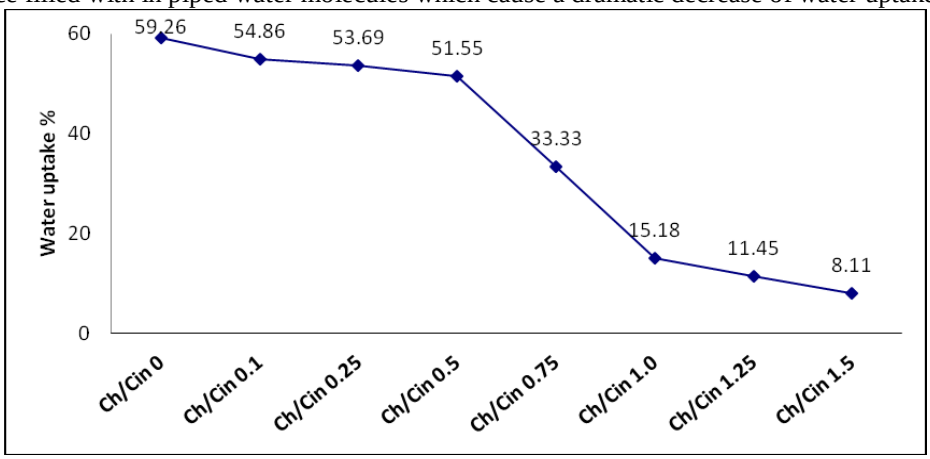

Figure 3: water up take of chitosan with different content of cinnamaldehyde.

3.1.3 Moisture content

Moisture content of chitosan membranes with different content of cinnamaldehyde were measured and presented in Figure 4. It is clear from the figure that the decreases of moisture content trapped in membranes by increase the content of cinnamaldehyde which may be explained by switching of membranes nature to be hydrophobic by adding cinnamaldehyde that reflected on its adsorption capacity of moisture from surrounding.

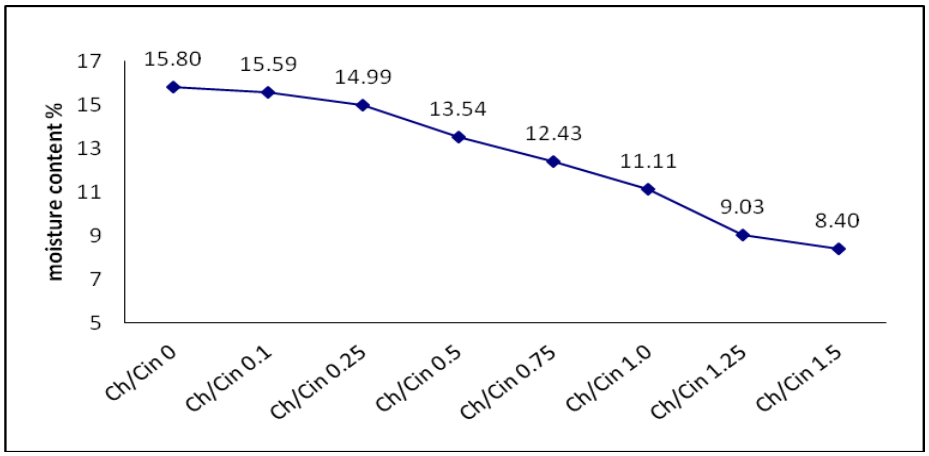

Figure 4: Moisture content of chitosan membranes with different content of Cinnamaldehyde.

3.1.4 Roughness

Surface roughness of the chitosan – cinnamaldehyde membranes Schiff bases were measured (Figure 5). Obtained results demonstrate increase in surface roughness of membranes that leads to increase of membranes surface area.

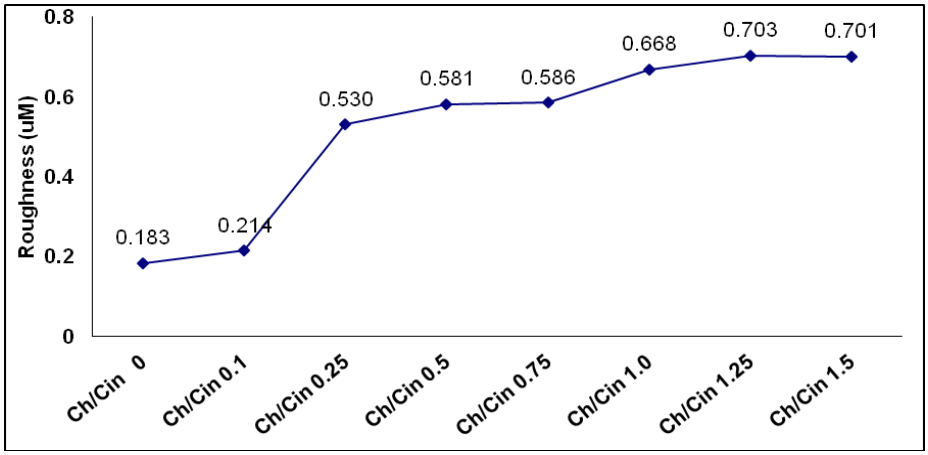

Figure 5: Surface roughness of chitosan membranes with different content of Cinnamaldehyde.

In fact, presence of new hydrophobic groups on chitosan chains affects the arrangement of molecules during casting process which led to disorder.

3.1.5. Wettability

Water contact angle with chitosan membranes was measured with advanced Gonimeter model 500 F1 (Table 1). There are increases of the contact angle by increasing the cinnamaldehyde content of membranes. This can be explained by increasing hydrophobic character of membranes.

Table 1: Contact angle of water on chitosan membranes with different contents of cinnamaldehyde.

	θ_R	θ_L	θ_{mean}
Ch/Cin 0	76.95	78.21	77.58
Ch/Cin 0.1	84.8	82.18	83.49
Ch/Cin 0.25	85.86	86.2	83.49
Ch/Cin 0.5	87.04	88.28	87.66
Ch/Cin 0.75	90.08	89.59	89.83
Ch/Cin 1	90.47	91.66	91.07
Ch/Cin 1.25	93.19	96.41	94.8
Ch/Cin 1.5	95.22	96.57	95.9

3.2. Electronic spectra characterization

3.2.1 Color measurement

Transparency of wound dressing membranes is important factor for follow up wound healing. Film absorption of lights was tested starting from 190 to 1100nm Figure (6). Increase of intensity and shift of bands at 320 of chitosan to red shift was attributed to lowering of energy of n- σ* transition and generation of new bands at 402 was attributed to n-π* transition. **[Perrin et al., 1980; Morrill et al.,1974; Mohy Eldin et al., 2015].**

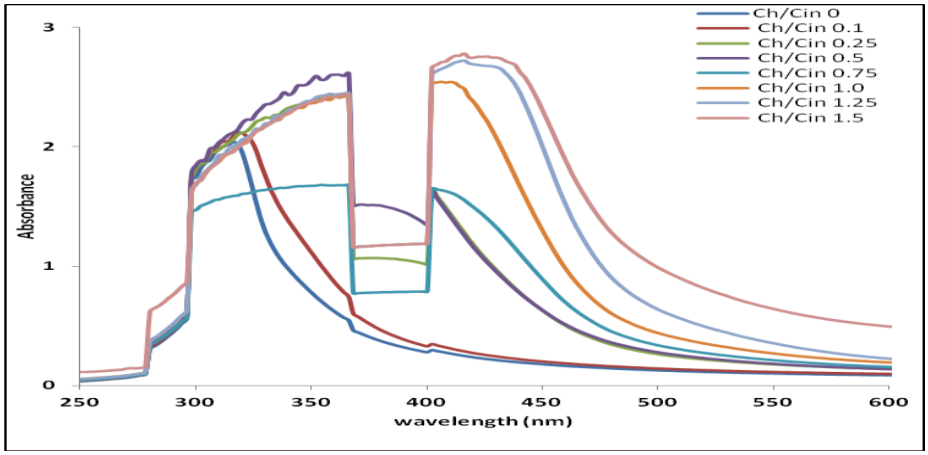

Figure 6: electronic spectrum of chitosan and chitosan schiff base membranes

Transparency of tested membranes was calculated and recorded in Table 2. It was shown that there is a decreasing in transparency and increase on the membranes thickness, and there is a direct result of coupling between free amino groups of chitosan and cinnamaldehyde.

Table 2: Thickness and transparency of chitosan membranes.

	thickness	A_{600}	T_{600}
Ch/Cin 0	0.05	0.084	39.87
Ch/Cin 0.1	0.054	0.096	36.9
Ch/Cin 0.25	0.057	0.139	34.82
Ch/Cin 0.5	0.062	0.137	31.98
Ch/Cin 0.75	0.071	0.159	27.83
Ch/Cin 1	0.075	0.191	26.23
Ch/Cin 1.25	0.086	0.225	22.62
Ch/Cin 1.5	0.091	0.493	20.99

Color of film is an important index in terms of general appearance and consumer acceptance. Rectangular coordinates (L, a, and b) and total color difference (Δ E) were calculated and are summarized in Table 3.

Table 3: Color vales of chitosan membranes with different content of cinnamaldehyde.

	L*	a*	b*	ΔE
Ch/Cin 0	85.66	2.47	15.3	36.17
Ch/Cin 0.1	81.76	3.06	19.49	31.67
Ch/Cin 0.25	81.55	2.6	24.38	31.49
Ch/Cin 0.5	79.4	9.77	56.38	44.84
Ch/Cin 0.75	73.99	12.35	58.6	43.67
Ch/Cin 1	71.88	16.15	55.37	39.23
Ch/Cin 1.25	70.17	19.91	59.86	44.61
Ch/Cin 1.5	67.34	22.85	59.4	44.1

Results show a decrease of surface brightness (L value) and increase in yellow color (b values). In fact brightness of membrane is a result of its crystal structure. Increasing of membrane disorder leads to decrease in its brightness, where the high shift of b value at high concentration of cinnamaldehyde is a result of coupling reaction between chitosan and aldehyde to form Schiff base that give absorbance at yellow region.

3.2.2 Scan Electron Microscope

Surface morphological examination of chitosan membranes with different contents of cinnamaldehyde was performed with Scanning electron microscope. Figure 7 show microstructure of membrane surface at different focusing powers. From figures it is clear that there is an increasing in surface roughness by increasing content of cinnamaldehyde, and this result is confirmed with results obtained from surface roughness measurement.

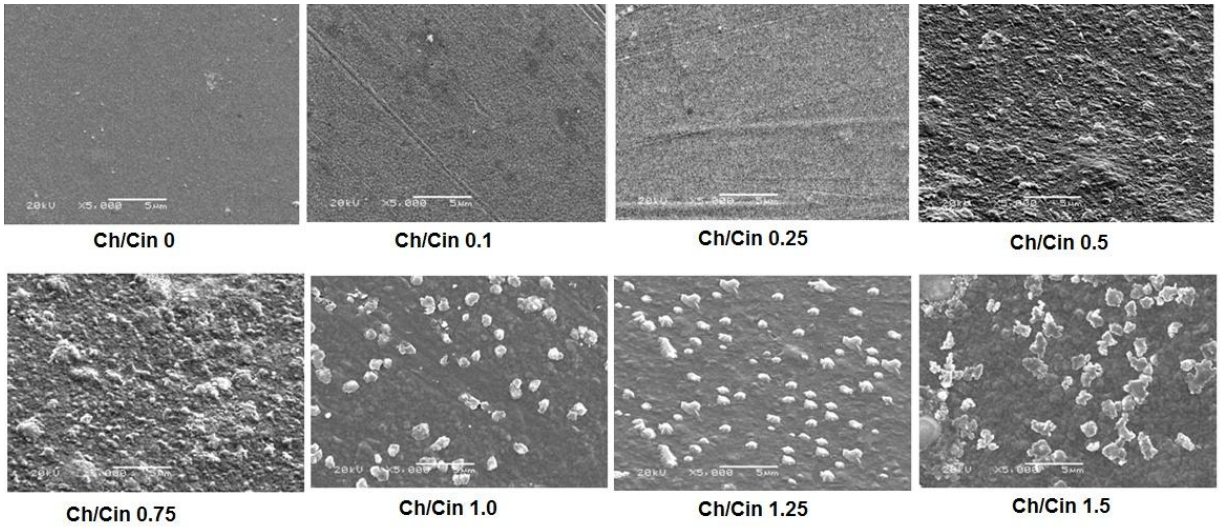

Figure 7: Scan electron microscope of chitosan and chitosan Schiff base membranes

3.3. Thermal analyses

3.3.1. Thermal gravimetric analysis

Thermal gravimetric analysis of eight membranes of chitosan and chitosan/ cinnamaldehyde Schiff bases was performed. Data of TGA are listed in Table 4 and illustrated by Figure 8.

Table 4: thermal gravimetric peaks of chitosan and chitosan Schiff base.

	Ambient-150 ^{o}C (%)	220-350 ^{o}C	T_{50}	T_{75}
Ch/Cin 0	8.08	39.66	411.3	794.72
Ch/Cin 0.1	12.13	37.78	376.63	507.74
Ch/Cin 0.25	12.9	36.9	377.54	662.3
Ch/Cin 0.5	11.04	35.58	429.26	676.03
Ch/Cin 0.75	12.41	35.25	409.59	632.16
Ch/Cin 1	12.74	37.36	370.36	589.95
Ch/Cin 1.25	10.46	41.65	324.67	542.65
Ch/Cin 1.5	6.7	35.21	452.48	617.17

Results indicate decrease in thermal stabilities of chitosan membranes by substitutions. In fact first transition of chitosan TGA from ambient temperature to about 150 °C is a result of elevation of moisture content attached to samples. Depression of weight at this temperature confirms physical adsorption of water molecules in between chitosan chains. It was observed that there is a general decrease in water moisture in samples which a direct result of decrease of material hydrophilicity by coupling cinnamyl nucleus to chitosan backbone.

A general decrease of thermal stability of chitosan can be shown in weight loss between 220-380 °C. At this temperature, initiative degradation of chitosan pyranose ring was started by some radical cleavage of it to form a crosslinked intermediate. **[Pawlak and Mucha., 2003].** Stabilization of the intermediate products by cinnamyl nucleuses of Schiff base enhances reaction to be at lower temperature.

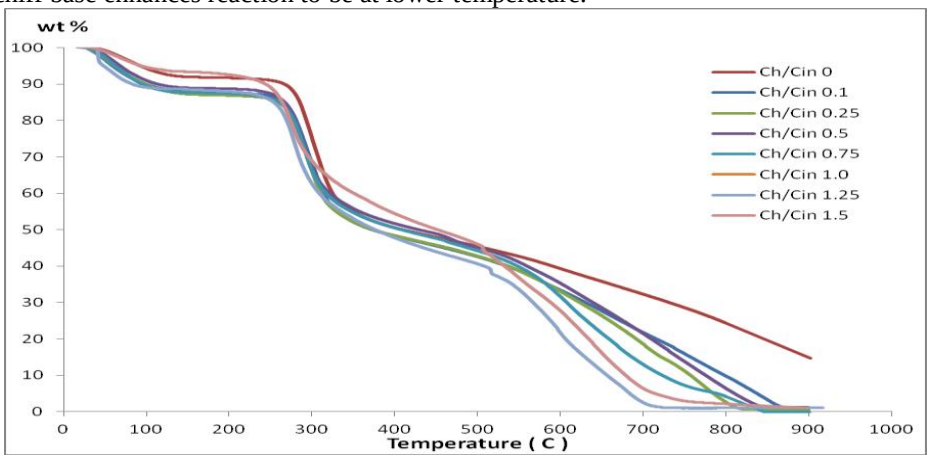

Figure 8: Thermal gravimetric analysis of chitosan and chitosan schiff base membranes

3.3.2. Differential scanning calorimetric (DSC)

Differential scanning calorimetric analysis of the prepared membranes was measured and presented as shown in Figure 9. Table 5 summarizes the temperature of both endothermic and exothermic peaks of membranes.

Table 5: Exothermic and endothermic peaks of chitosan and chitosan Schiff base membranes.

	1st endothermic (°C)	2nd endothermic (°C)	3rd endothermic (°C)	1st exothermic (°C)	2nd exothermic (°C)	3rd exothermic (°C)
Ch/Cin 0	101.94	267	---	168.1	---	---
Ch/Cin 0.1	97.16	265.56	---	173.16	---	---
Ch/Cin 0.25	104.02	260.97	---	178.19	---	---
Ch/Cin 0.5	109.11	245.57	---	172.5	284.41	---
Ch/Cin 0.75	102.79	251.93	---	168.05	287.65	---
Ch/Cin 1	97.9	212.09	289.42	142.35	272	292.23
Ch/Cin 1.25	89.45	226.81	289.6	136.98	269.57	291.57
Ch/Cin 1.5	93.18	212.86	282.8	139.42	269.8	284.79

As mentioned before, first endothermic peak observed in between 90-110 °C is attributed to moisture elevation which is attached on internal chains. Second endothermic observation was shown in between 212 – 265 °C which results from cleavage of the glycoside ring. Increase of thermal stability of substituted chitosans rather than chitosan itself can be explained by stability power of cinnamaldehyde groups to the free radical fragments that resulted from thermal degradations.

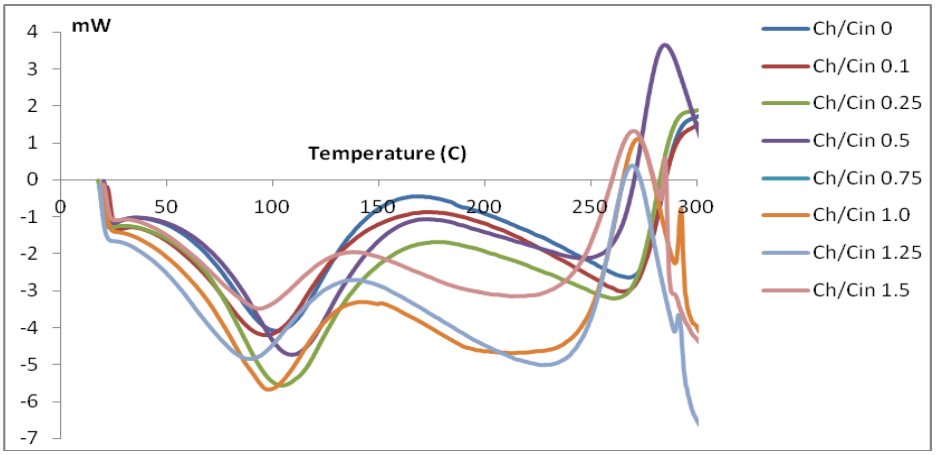

Figure 9: Differential Scanning Calorimetric (DSC) of chitosan and chitosan schiff base membranes

3.4. Mechanical properties

Mechanical properties of chitosan membranes with different contents of cinnamaldehyde were determined from critical breaking point of stretching pieces maximum stress σ_{max} (Nm^{-2}) was evaluated as the ratio of the stretching force divided by the cross-sectional area of broken membrane piece. The maximum strain λ_{max} was measured as the elongation ratio of the initial length of the test piece. Results obtained are listed in Table 6.

From the results it can be seen that there is a general decrease in all parameters by increasing cinnamaldehyde content. In fact, presence of high number of cinnamaldehyde substituted to chitosan chains impairs mechanical properties. This effect is related to the formation of lower crosslinked network between chains (amine hydrogen bonds) due to presence of bulky groups.

Table 6: Mechanical parameters of chitosan and chitosan Schiff base membranes.

	Max stress σ_{max} (N/mm^2)	Max strain λ_{max} %	Energy (J)	break Displacement (mm)
Ch/Cin 0	64.08	20.53	0.441	8.7
Ch/Cin 0.1	59.99	9.84	0.315	4.5
Ch/Cin 0.25	57.34	7.27	0.202	3
Ch/Cin 0.5	58.28	4.72	0.054	1.415
Ch/Cin 0.75	62.78	3.68	0.075	1.842

Ch/Cin 1.0	38.28	2.18	0.025	0.873
Ch/Cin 1.25	29.65	1.55	0.017	0.621
Ch/Cin 1.5	1.70	1.02	0.0005	0.305

3.5. Membrane bioevaluation

3.5.1. Haemocomptability of membranes

Several essential requirements must be taken into consideration during preparation and qualification of medical devices especially blood contact materials. Blood compatibility is recognized to play important parameter during evaluation of wound dressing membranes. Value of Haemolysis is taken as a mentor test. Figure 10, illustrates haemolysis percent of prepared membranes. Haemolysis is regarded as an especially significant screening test. Once it provides quantification of small levels of plasma hemoglobin, which may not be measurable under in-vivo conditions. As reported in literature (ISO 10993-4(1999)), it is not possible to define a universal level of acceptable or unacceptable amounts of haemolysis. Although by definition a blood compatible materials should be non haemolytic, in practice several medical devices cause haemolysis. This means that when such haemolytic effect takes place, it is important to make sure that clinical benefits overcome these risks and that the values of haemolysis are within acceptable limits.

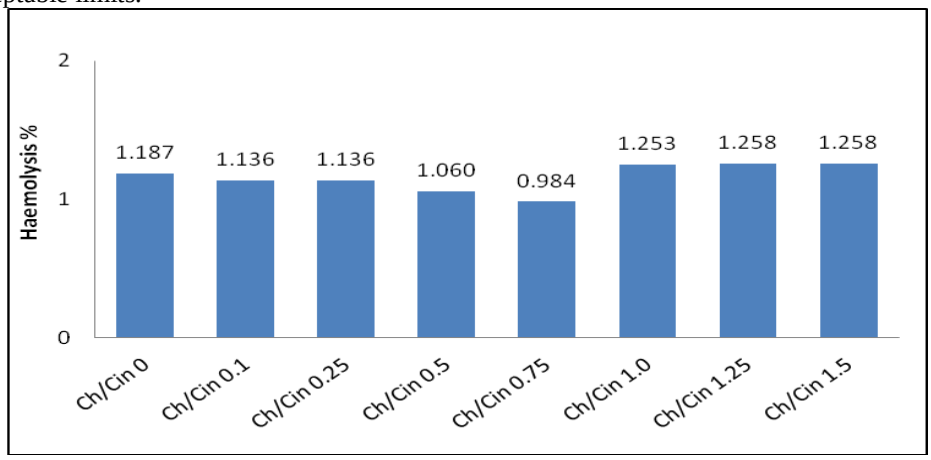

Figure 10: Haemolytic percent of chitosan and chitosan Schiff base membranes

According to ASTM F 756-00 (2000) materials can be classified in three different categories according to their haemolytic index (haemolysis %): materials with percentages of haemolysis over than 5% are considered haemolytic; while the ones with haemolysis index between 5% and 2% are classified as slightly haemolytic. Finally, when material presents a haemolysis percentage below 2%it is considered as a non haemolytic material.

From figure 10, it is evidence that there is a decrease of haemolysis percent by modification. In general results obtained indicate that chitosan and chitosan Schiff base membranes are non haemolytic.

3.5.2. Blood clotting index

Another important test in going to evaluate wound dressing membranes is to measure its ability to accelerate haemostatic action. Figure 11, shows effect of modification of chitosan with cinnamaldehyde on blood clotting index when they are in contact with blood samples. Results show an increase of clotting index by modification.

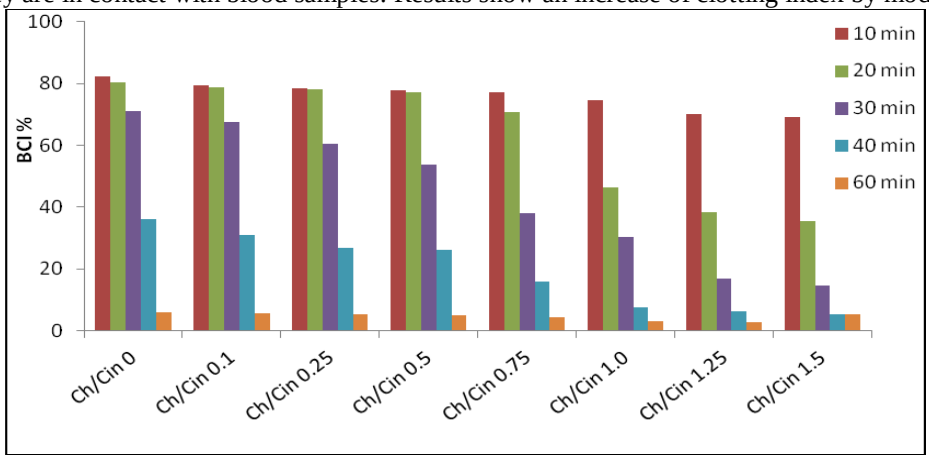

Figure 11: blood clotting index of chitosan and chitosan- cinnamaldehyde Schiff base membranes.

This may be explained by fast adsorption process of blood protein that is responsible for clotting process initiation step.

3.5.3. Thrombogenicity of membranes

As membrane is designed to be used topically in wound healing, it is important to evaluate its tissue and blood compatibility. Furthermore, thrombogenic character is a desirable property in membranes. Once the protein adhesion constitutes the first step to initiate coagulation cascade, membranes can accelerate the thrombus formation, stopping haemorrhage and helping in healing process. Fig. 12 shows the weights of blood clots obtained on thrombogenicity test. It was observed that clot formation was lower in membranes than in the control and for this reason, the polymers are classified as non-thrombogenic **[Imai and Nose., 1972]**. This characteristic is directly related to the hydrophilicity of the materials. When placed in contact with a hydrophobic surface, proteins adsorb to it in a strong and irreversible way, while at hydrophilic surfaces proteins adsorb weakly and reversibly **[Changsheng et al., 2003**]. This relation between hydrophilicity and thrombosis was confirmed by the lower value of thrombus weight that was formed when blood contacted with chitosan membranes and that will increase by cinnamaldehyde substitution as a direct result of increase of hydrophobicity.

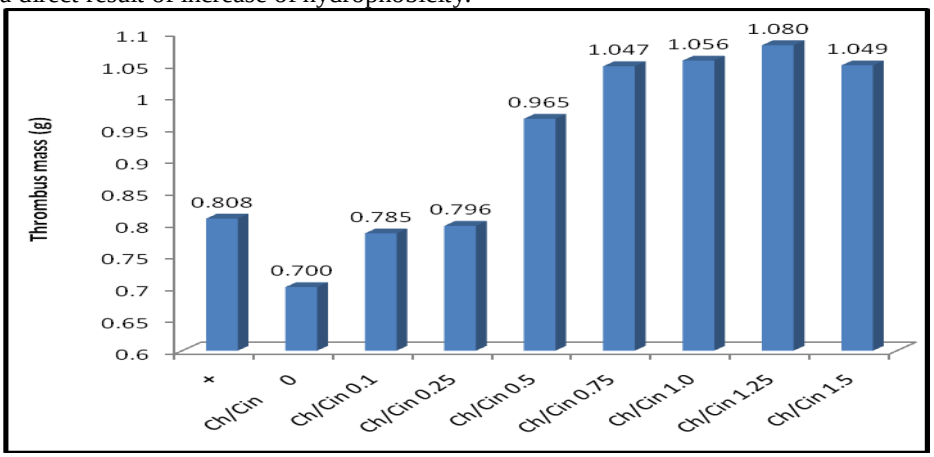

Figure 12: Thrombogenic index of chitosan and chitosan –cinnamaldehyde Schiff base membranes

3.5.4. Protein adsorption

Adsorption of proteins on membranes surface is considered one of the essential measurement to evaluate it in field of wound dressing applications. In fact, adsorption of blood protein initiates process of blood clotting. Figure 13 shows adsorption of bovine serum albumin on surface of chitosan and cinnamyl chitosan membranes. It is evident that there is a dramatic increase of protein adsorption by increasing substitutions which is attributed to increase of hydrophobicity. Both protein and membrane surface have non-polar groups which are exposed to aqueous solution. Gibb's energy ($\Delta adsG=\Delta adsH-T\Delta adsS < 0$) decreases when the hydrophobic regions of sorbent and protein get dehydrated (predominantly due to an increase in entropy of the liberated water molecules). Hydrophobicity of protein exterior influences protein adsorption at solid/water interfaces. In general, protein molecules change their conformations to a large extent on hydrophobic surfaces than on hydrophilic surfaces. This is because the hydrophobic part of protein and hydrophobic part of surface interact together.

Experiments, which used a surface with a hydrophobic gradient, show that on hydrophobic side of surfaces adsorbed amount is always higher than on hydrophilic side (but a study by Veen **[van der Veen et al., 2006]** has showed that at high concentration adsorbed amount is higher at hydrophilic surface (concentration effect).

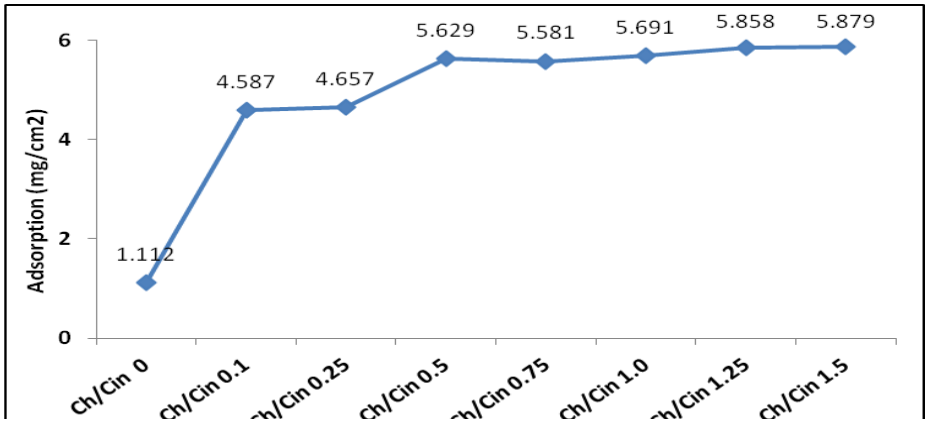

Figure 13: Protein adsorption index of chitosan and chitosan –cinnamaldehyde Schiff base membranes

Marie Wahlgren and Thomas Arnebrant (1991) studied the adsorption process for fibrinogen, which is classified as a hard protein. It was found that adsorption on a surface with a hydrophobicity gradient decreased with decreasing water contact angle. At the same time the desorbability on the hydrophobic side decreased with time, likely reflecting a slow increase in the hydrophobic interactions of protein with the sorbent surface. **Krisdhasima et al. (1992)** observed same behavior for adsorption of β-Lg (hard) on hydrophobic silicon. It was found that increasing hydrophobicity leads to increasing adsorbed amounts. HPA, which is a soft protein, still adsorbed on oxide at high pH values. Under these conditions electrostatic forces are repulsive and dehydration of the surface is also unfavorable. So other forces like the properties of protein such as hydrophobic residues are governing the adsorption process. Hydrophobic proteins, which are exposed to water, are energetically unfavorable because the enthalpy of this interaction is small and negative but water gets hydrated on the hydrophobic part of the protein (loss of entropy of water molecules) and as a consequence the Gibbs free energy will increase. If a protein interacts with a surface, the energy of the system will be minimized because water gets released from the surface and the protein (gain of entropy).

An interaction with a hydrophobic surface is energetically more favored than with a hydrophilic surface because water molecules are released from the surface and from the protein, which leads to a large entropy gain. **[Krisdhasima et al., 1992; Paul Roach et al., 2005].**

3.5.5. Biodegradability

Biodegradability of chitosan and cinnamyl chitosan membranes was determined by reducing sugar measurement to estimate the concentration of glucose amine (chitosan monomer) residue. The results of our biodegradation testing are shown in Figure 14. Figure monitoring the concentration of reducing sugar of solution in presence and absence of lysozyme. In the presence of lysozyme a dramatic increase of degradation rate until Ch/Cin 0.5 was observed. At high substitution, the degradation rate decreases. This can be attributed to strict effect facing the enzyme receptors as a result of increased substitution.

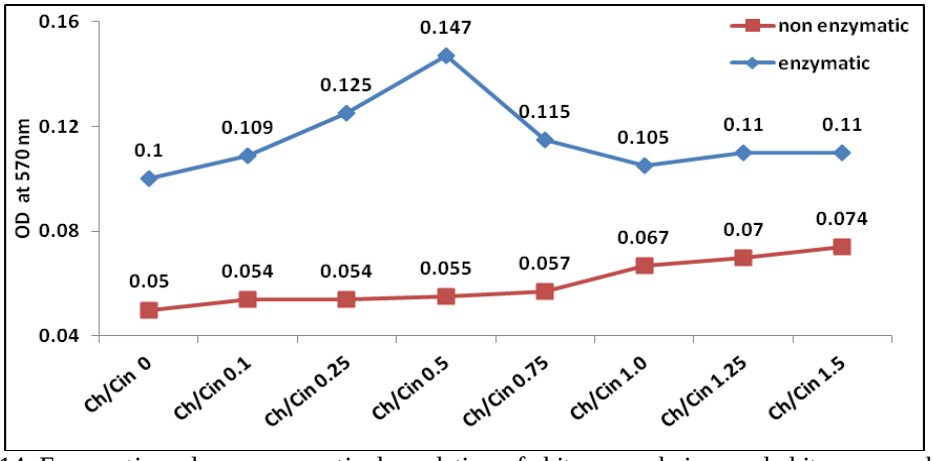

Figure 14: Enzymatic and non-enzymatic degradation of chitosan and cinnamyl chitosan membranes.

It is well known that, in human serum, N-acetylated chitosan is mainly depolymerized enzymatically by lysozyme, and not by other enzymes or other depolymerization mechanisms **[Smidsrod et al., 1997]**. The enzyme biodegrades

the polysaccharide by hydrolyzing the glycosidic bonds present in the chemical structure. Lysozyme contains a hexameric binding site **[Pangburn et al., 1982]**, and hexasaccharide sequences containing 3–4 or more acetylated units contribute mainly to the initial degradation rate of N-acetylated chitosan **[Smidsrod et al., 1997].**

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