ELABORATION AND PHYSICAL, CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF NEW CHITOSAN AND GELATIN MEMBRANES

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The paper is focused on elaboration and physical chemical and biological characterization of new membranes based on chitosan and gelatine. The membranes were prepared using two gels chitosan and gelatin, in molar ratio 1:1 and 1:3. The surface characterization includes scaning electronic microscopy (SEM),, water absorbtion, and determination of contact angles. As biological tests hemolysis and biodegradation were performed.

Keywords: chitosan, gelatine, contact angles, biodegradation, haemolysis

1. Introduction

Due to its antimicrobial, non-toxic, biocompatible and biodegradable properties [1] **chitosan,** a cationic natural polymer, has been widely used as a topical dressing in wound management owing. **Gelatin** is the product of thermal denaturation of insoluble collagen [2] with various molecular weights (MWs) and isoionic points depending on the source of collagen and the method of its manufacturing process of recovery. Collagen exists in many different forms, but gelatine is only derived from source rich in Type I collagen. Gelatine was employed as biomaterials more recently [3]. Many materials have been used to improve the chitosan membrane, such as nanowhiskers [4] PVA-poly-(vinyl alcohol) [5], or collagen [6]. Being a component of bone collagen is well known

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as a main component of extracelular matrix as well. Biomaterials, such as Chitosan and Gelatin, are frequently used in prosthetic devices designed to replace or support failing tissues or organs in patients having a friendly response in the surrounding tissues in terms of biocompatibility. As a demerit of chitosan properties it is to mentioned its poor mechanical properties and low thermal stability, which limits its practical application.
Blending of chitosan with other polymers and crosslinking are both convenient and effective methods of improving the physical and mechanical properties of chitosan in bioapplications [7, 8]. The present paper is focussed on such biomaterials which present a special feature in water adsorption phenomena, interstingly exploited in bioapplications as dressings, adsorbent, supports for the gradual release of drugs or used as biocompatible coatings for implants.

2. Materials and membrane characterization

*2.1 Materials***: Chitosan** (CHI) obtained from crab shells, with molecular weight M=150,000 and degree of deacetylation DD = 84.5% (Fluka BioChemika), viscosity high, <12% loss on drying (Sigma Aldrich). **Gelatine** (GEL) the dermis and tendons of**, (**Fluka BioChemika). **(**Merck), **Bacterial collagenase** *Clostridium histolyticum*, E.C. 3.4.24.3. (Sigma), Sodium acetat, acetic acid were all of reagent grade.

a) Preparation of chitosan gel :

1% chitosan gel was prepared using 1 g of chitosan diluted in acetic solution (acetic acid 2M and sodium acetat 1M) and stirred with the magnetic agitator at 50ºC till a homogenous gel (pH=5.4) was obtained.

*b) Preparation of gelatin gel***:**

1 gram of gelatin powder was soaked in 100 mL deionized water and heated at 60°C until a homogenous gel was obtained. The solubility of gelatin in aqueous solutions is temperature dependent. The appropriate mass of gelatin was added to the appropriate volume of deionized water at ultimately, the gels is prepared for immediate use. In the case where there is left over gels, it is stared in a refrigerator and headed before use.

*c) Elaboration of biopolimeric membranes***.** Four types of samples were prepared as following: a: CHI; b**:** GEL; c: CHI:GEL (1:1), d: CHI:GEL (1:3),

As references chitosan and collagen membranes have been fabricated.

After stirring vigurously for 30 minutes, the mixtures were placed in Petri vases and heated at 37°C, for 48 hours. Finally semitransparent and semielastic membranes have been obtained.

2.2. Membrane characterization.

*a) Fourier Transmission Infra-Red Spectroscopy (FTIR***)**

The FTIR spectrum of the chitosan-gelatin membranes was obtained using a FTIR analysis using a Perkin Elmer Spectrum GX (FT-IR system). All spectra were recorded within a range of 4000–500 cm-1 with a 4 cm-1 resolution. All measurements were performed in a dry atmosphere at room temperature.

b) Microscopy investigation: Scanning Electron Microscopy (SEM)

For microscopic investigation a SEM Hitachi SU1510 was the equipment of the surface characterization of Chitosan-Gelatin films.

c) Swelling Behavior (E_s)

The swelling behavior was investigated using rectangular samples of ~ 20 mm x 20 mm by a gravimetric method. Each sample, after submersion in phosphate buffer saline solution (PBS-pH 7.4), (0.8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4 $2H_2O$ and 0.2 g KH_2PO_4 dissolved in 1 L of distilled water; pH 7.2) for 24 hours, was taken out and placed between two pieces of absorbent paper to remove excess PBS. The degree of swelling (%) was calculated according to:

$$E_s = \frac{W_s - W_D}{W_D} \cdot 100$$

where: E_S is the equilibrium Swelling ratio

W_S and W_D denote the weights of swollen and dry samples, respectively**.**

d) Contact angle measurements

The hydrophilic/hydrophobic balance of synthesized membranes was evaluated by measuring the static contact angle of a drop of water deposited on the membranes surface. The contact angle **(**CA**)** of a drop of water with the membranes surface was measured as previously described [9, 10] with a contact Angle Meter, KSV instruments CAM 100 equipment. The water contact angle measurements were conducted at room temperature. Distilled water was used at the wetting liquid. Each experiment was conducted in triplicates and the mean values were computed.

e) In vitro membranes biodegradation

In vitro biodegradability of membranes have been determined in the presence of Bacterian Colagenase (*Clostridium histolyticum*, E.C. 3.4.24.3. Sigma) according to a protoccol established in the Department of Molecular and Celular Biology in the frame of National Institute of Research and Development for Biological Sciences, Bucharest. [11].

f) Hemolytic study

Hemostasis ability by fragility of RBC membranes in contact with absorbable gelatin sponge was investigated according to the ASTM-F75600 [12]. For hemolytic study an anticoagulant blood was prepared by adding 1 mL of

anticoagulant acid citrate dextrose solution (ACD) to 9 mL of fresh blood. Biopolymeric membranes were cut into appropriate pieces (3 cm²) and then transferred to polyethylene tubes. One mL of whole blood was added to tube and diluted by 7 mL phosphate buffer. The solutions were then incubated at 37ºC for 72 min. After incubation, the PBS was removed and 1mL ACD blood (9.02 mg/mL) was added to each sample and maintained at 37°C for 3 h. Following this, they were centrifuged at 800 rpm for 15 min. The supernatant was then taken for hemolytic studies. Positive and negative controls were prepared by adding the same amount of ACD blood to 7mL of water and PBS, respectively. Absorbance at 540 nm for the bipolymeric membranes and control were determined by UV–VIS Jenway Spectrophotometer. The percentage of haemolysis was calculated using the equation described in a previous paper [13].

3. Results and discussions:

a) FTIR structure of all studied membranes can be seen in Fig.1.

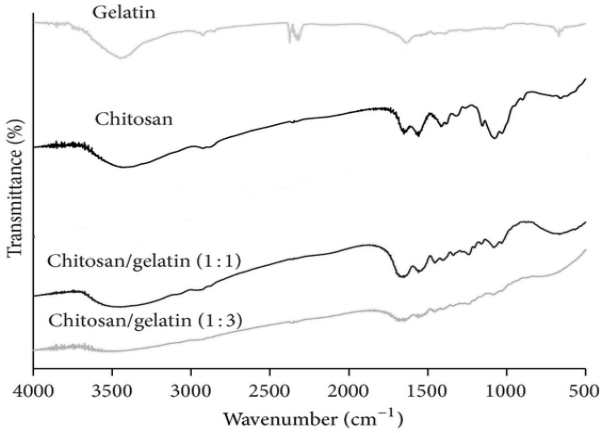


Fig.1. FT-IR spectra of membranes chitosan, gelatin and chitosan:gelatin

The IR spectrum of the chitosan has a peaks observed at 3454 cm^{-1} due to OH group and the band at 2876 cm^{-1} and 1026 cm^{-1}, indicated aliphatic C-H streaching and C-O-C stretching vibrations respectively [14]. On the other hand, the peak at 1643 cm^{-1} has been attributed to C=O stretching (amide I). The band at 1584 cm^{-1} was assigned for NH bending for the NH_2 groups on chitosan. [15]. The peaks observed at 1419, 1376, and 1318 cm^{-1} may be due to C-H stretching.

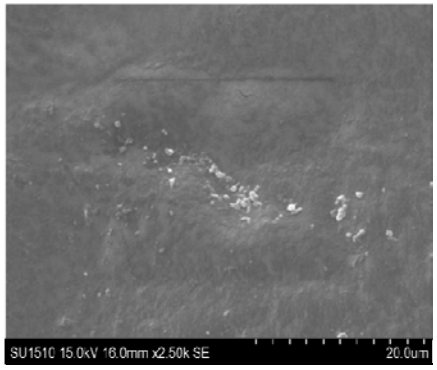
Gelatin film revealed absorption bonds at 3285 cm^{-1} corresponding to NH stretching vibration 3085 cm^{-1} for alkyl C–H stretch, 2956 cm^{-1} for CH_2 asymmetrical stretching, 1631 cm^{-1} for C=O stretch/HB coupled with COO−,

band at 1533 cm^{-1} for N–H bend coupled with CN stretch, 1444 cm^{-1} for CH_2 bend, 1240 cm^{-1} for NH bend, and 1078 cm^{-1} for C–O stretch. [16].

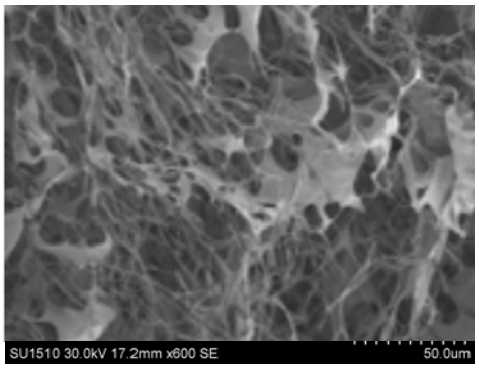
The FT-IR of composite membrane exhibited a mixture of characteristic absorptions due to the amine groups of chitosan and the carboxylic acid groups of gelatin. The peaks of amide I for chitosan at 1643 cm^{-1} and 1584 cm^{-1} was shifted to 1639 cm^{-1} and 1537 respectively in composite membranes. In composite membranes spectra the amino band at 1533 cm^{-1} characteristic for gelatin shifted to 1634 cm^{-1}, and a carbonyl peak shifted to 1537 cm^{-1}. The intensity of C = O peaks increased with the decline ratio of gelatin in composite membranes from 1:3 to 1:1 (chitosan/gelatin). These results can be attributed to the interaction of $-NH_3^+$ of chitosan and the COO− of gelatin. [17].

b) SEM

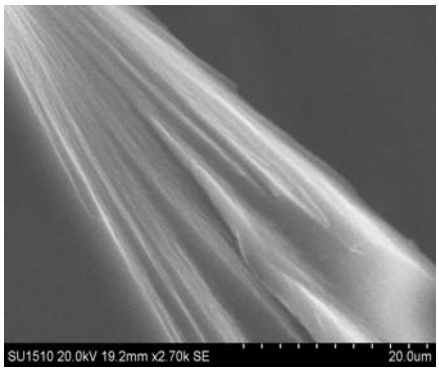
In general, porosity, pore size and orientation of porous scaffold were indispensable elements of biological activity of biomaterials having an open-pored structure. The surface topography of membranes are illustrated in Fig 2.



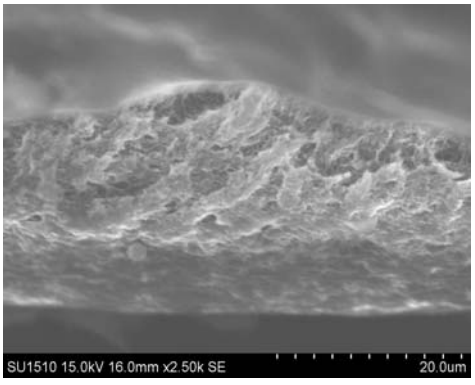
Chitosan (a)



Gelatin (b)



CHI:GEL (1:1) (c)



CHI:GEL (1:3) (d)

Fig. 2. SEM micrographs of membranes

Fig. 2a shows the SEM micrograph of pure chitosan membranes. The morphology shows a smooth surface because the sample was prepared in thin film form. The morphology shows some small precipitate on the specimen which may be some un-dissolved chitosan or chitosan crystal.

The SEM of gelatin membrane (b) showed fiber-like structure. For chitosan:gelatin membranes (c, d) showed membrane-like structure with interconnected pores.

c) The equilibrium Swelling ratio (E_S)

The swelling ability of membranes plays an important role during in vitro culture. When the membranes was capable of swelling, it allowed the pore sizes to increase in diameter thus facilitating he cells not only to just attach but also to migrate inside the membranes and grow in a three dimensional fashion, during in vitro culture studies.

The swelling studies of the membranes in PBS were shown in Fig. 3.

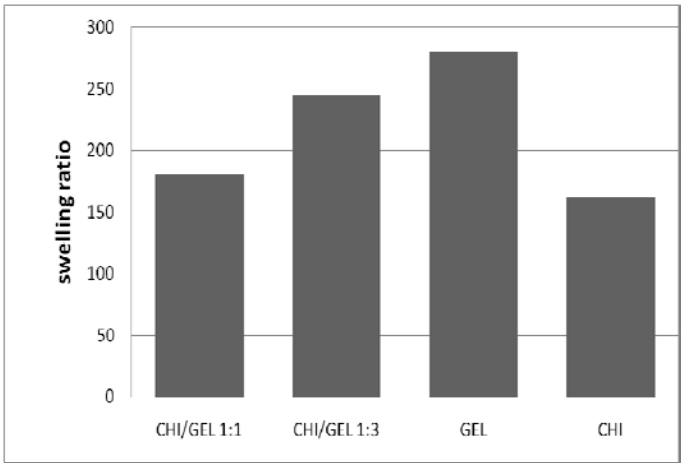


Fig.3. Swelling ratio of the membranes in PBS

The high water content in prepared sponges was explained by the hydrophilic and swelling properties of gelatin [18].

d) Contact angle measurements:

Table 1 showed that contact angle of all membranes. There was significant difference between contact angle values of pure chitosan membranes and chitosan-gelatin 1:3. Contact angle values decreased with increasing content of gelatin membrane. The decrement of water contact angle confirms the successful hydrophilic modification on the support membrane enrichment of gelatin to the membrane surface. Roughness is high for membranes with GEL.

Table 1

Contact angles and roughness values for biopolymeric membranes

Sample/composition	Roughness	CA
GEL gels	86	32.37
CHI gels	36	64.01
CHI-GEL (1:1)	64	50.07
CHI-GEL, (1:3)	58	48.56

It is worth noting that films with higher moisture contents had lower contact angles, indicating more ability to absorb water and so that higher hydrophilicity. Therefore, contact angle results were in good agreement with the results obtained in the swelling ability.

e) Membranes biodegradability evaluation *in vitro*

The biodegradability of biomaterials such as chitosan-gelatine membranes, plays an important role in tissue engineering. This process can affect many processes including tissue regeneration and host response [19].

Data about elaborated membranes biodegradability are presented in Fig.4.

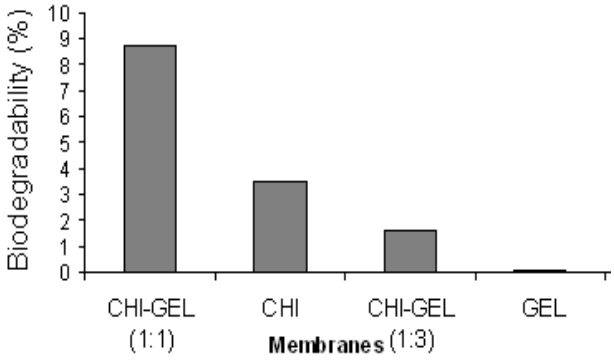


Fig. 4. Membranes biodegradability *in vitro.*

The results have indicated a high biodegradability level as 8,76 %. for membrane chitosan-gelatine.

f) Hemolityc studies

Hemolysis 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. According to ASTM F 756-00 (2000) materials can be classified in three different categories according to their

hemolytic index (hemolysis %). Materials with percentages of hemolysis over 5% are considered hemolytic; while the ones with hemolytic index between 5% and 2% are classified as slightly hemolytic. Material which a presents a hemolysis percentage below 2% it is considered as a non-hemolytic material.

Table 2

The hemolysis index values for all membranes.

Samples	Haemolityc percent
Chitosan	2,64
Gelatin	0.86
Chitosan-gelatin 1:1	1.68
Chitosan-gelatin 1:3	1.12

The results indicate that the chitosan membranes are slightly hemolytic and chitosan-gelatin membranes are non hemolytic compounds and compatible with human body. Addition of gelatine on chitosan membranes decreased the hemolytic index to less than 2%, so the presence of gelatine is beneficial from this point of view. The reduction of hemolytic effect is directly proportional with surface roughness mentioned in table 1.

4. Conclusions:

Based on experimental data, a comparison between structure and properties of membranes as a function of ratio chitosan gelatin have been discussed. The paper presented a method for elaborating and characterizing chitosan-gelatine membranes with various ratios of chitosan and gelatin. The structure and morphology were characterized using FTIR and SEM. The effect of increase of gelatine addition in membranes may be explained according to reduce the surface roughness and accordingly the contact area between the blood and the membranes surface. The increase of gelatin content improved the swelling ration contact angle, and hemolysis index recommending this membrane as a better choice for cell response.

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R E F E R E N C E S

[1]. *T.Dai, M. Tanaka, Y.-Y. Huang, M. R. Hamblin,* Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. Expert Rev. Anti Infect THER, **vol. 9**, no. 7., 2011. pp 857-879.

[2]. *A.Mauthe,D.Hutchison, G.Coulson and S.Namuye*,Multimedia Group Communications Towards New Services", in Distributed Systems Eng., **vol. 3**, no. 3, Sept. 1996, pp. 197-210.

[3] *M.C.Gomez-Guillen, M.Perez–Mateos, J.Gomez-Estaka, E.Lopez-Caballero, B.Gimenez and P. Montero,* Fish gelatin: a renewable material for developing active biodegradable films trends in Food Science & Technology, **vol 20,** no.1, 2009, pp 3-16.

[4]. *S. Georgieva and V. Kokol,* Collagen-vs Gelatine Based biomaterials and their Biocompatibility. Rewiev and perspectives. Biomaterials Applications for Nanomedicine. Edited by prof. Rosario Pignatello, ISSN 978-953-307-661-4.

[5]. *JoP de Mesquita, C.L. Donnici, F. V. Perreira,* Biobased nanocomposite from layer-by-layer assembly of cellulose nanowhiskers with chitosan. Biomacromolecules. **vol. 11,** 2010, pp. 473-480.

[6]. *Y.Zhou D.Yan, X.Che, Q.Xu, F.Lu, J.Nie,* Electrospun water soluble carboxyethyl/ chitosan/poly (vinyl alcohol) nanofibrous membrane as potential wound dressing for skin regeneration. Biomacromolecules.; **vol. 9,** 2007, pp. 349-354.

[7]. *Y.Wu, Y. Hu, J.Cai, S. Ma, X.Wang,* Coagulation property of hyaluronic acid–collagen/chitosan complex film, Journal of Materials Science: Materials in Medicine, **vol. 19**, 2008, pp. 3621-3622.

[8]. *E. Mirzaei, A. Ramazani, M. Shafiee , M. Danaei*, Studies on Glutaraldehyde Crosslinked Chitosan Hydrogel Properties for Drug Delivery Systems. International Journal of Polymeric Materials and Polymeric Biomaterials, **vol 62**, 2013, pp: 605-611.

[9]. *A.R. Sarasam, R.K. Krishnaswamy, S.V. Madihally*, Blending chitosan with polycaprolactone: effects on physicochemical and antibacterial properties, Biomacromolecules. **vol 7**, 2006, pp 1131-1138.

[10]. *T.G.Tihan, S.Minkovska, M. P.Giurginca, T. Tite, H.Iovu, I.Demetrescu,* Hydrophilic/hydrophobic balance in relation with structure and biocompatibility of ternary biofilms PVA-HAP-Collagen gel, Molecular Crystals and Liquid Crystals, **vol. 486,** 2008. pp. 175/[1217] – 182/[1224].

[11]. *C.Tangsadthakun, S.Kanokpanont, N.Sanchavanakit, T.Banaprasert, S.Damrongsakkul,* Properties of collagen/chitosan scaffolds for skin tissue engineering, Journal of Metals, Materials and Minerals, **vol. 16,** no.1, 2006, pp. 37-44.

[12]. *L.Moldovan, O.Craciunescu, E.I.Oprita, M.Balan, O.Zarnescu,* Collagen-chondroitin sulfate-hydroxyapatite porous composites: preparation, characterization and in vitro biocompatibility testing. Roumanian Biotechnological Letters, **vol. 14**, no. 3, 2009, pp. 4459-4466.

[13]. Standard Practice for Assessment of Hemolytic Properties of Materials ASTM F756-00.

[14]. *A.Mazare, D.Ionita, G.Totea, I.Demetrescu, et al.,* Calcination condition effect on microstructure, electrochemical and hemolytic behavior of amorphous nanotubes on Ti6A17Nb alloy, Surface& Coatings Technology, **vol. 252,** 2014, pp. 87-92.

[15]. *Z.Osman, A.K.Arof,* FTIR studies of chitosan acetate based polymer electrolytes. Electrochimica Acta, **vol. 48**, 2003, pp. 993-999.

[16]. *M.F.Nazarudin, A.A.Shamsuri and M.N.Shamsudin,* Physicochemical Characterization of Chitosan/Agar Blend Gel Beads Prepared Via the Interphase Method with Different Drying

Techniques, International Journal of Pure and Applied Sciences and Technology, **vol. 3,** 2011, pp. 35-43.

[17]. *J.H.Muyonga, C.G.B Cole, K.G.Duodu,* Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (Lates niloticus). Food Chemistry. **vol. 86**, 2004, pp. 325-332.

[18]. *F.L.Mi*, Synthesis and characterization of a novel chitosan-gelatin bioconjugate with fluorescence emission. Biomacromolecules, **vol.6,** 2005**,** pp. 975-987.

[19]. *T.V.L.Hima Bindu, M.Vidyavathi, K.Kavitha, T.P.Sastry, and R.V.Suresh Kumar*, Preparation and evaluation of ciprofloxacin loaded chitosan-gelatin composite films for wound healing activity, International Journal of Drug Delivery, **vol. 2,** no. 2, 2011, pp 453-463.