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Development and sequential analysis of a collagen-chitosan wound management biomaterial

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Abstract

An electrospinning method was used to fabricate a bilayer collagen sponge/chitosan (Col/CS) matrix to develop a new wound healing dressing. The aim of the study was to improve the mechanical properties and antimicrobial activity of a collagen sponge matrix used as medical device. Col/CS matrix was subjected to detailed analysis by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), Atomic force microscopy (AFM), comparative physico-chemical characteristics (swelling ratio and hydrolysis properties, enzymolytic properties, bilayer Col/CS morphology) between collagen sponge and Col/CS matrix, bioburden determination and antimicrobial activity. The physical characteristics, morphology and antimicrobial properties showed that this biomaterial has a high ratio of surface area and superior mechanical properties, than collagen sponge. After sterilization, the recovery efficiency of S. aureus and E. coli from Col/CS matrix was 36% and 52%, result confirmed through qualitative antimicrobial activity. These results suggest that Col/CS matrix could be a promising solution for chronic wounds management.

Keywords: Collagen, Chitosan, Electrospinning, AFM, SEM, FTIR, Antimicrobial activity

1. Introduction

Since interruption or failure of wound healing process may easily lead to non-healing chronic wounds, wound management is increasingly becoming a vital factor in the process of wound healing. The basic requirement for all wounds to heal is a clean and adequate environment. This may require removal of non-viable tissue, control of bacterial burden, maintenance of moisture balance, and an environment which can adequately stimulate epithelialisation (M. FLANAGAN [1], K. VOWDEN & P. VOWDEN [2]).

Despite the fact that characteristics of an ideal dressing were identified many years ago, the perfect product has not yet developed, even though, each year, more products are launched into an

already crowded market. However, there is still a need for novel medical devices for wound management, because of the many interrelated factors that influence the selection of a proper wound dressing such as wear time, cost effectiveness, wound characteristics, as well as extrinsic factors related to patient, clinician, or the local health organization.

Wound care management has received much attention over the last two decades, leading to a substantial replacement of traditional wound dressing materials such as cottonwool, gauze and bandages, with new materials. These recently developed materials cover and protect the damaged tissue as a traditional wound dressing does, and beside that, they are designed specifically to offer improved healing rates by activating the cell proliferation and stimulating the healing process (L. O'NEILL & al. [3], C. VALENTA & al. [4]).

In this context, collagen-based dressings have been extensively studied over time (J.A.M. RAMSHAW & al. [5], S.H. LIU & al. [6], M. MIAN & E. BEGHE [7]). Collagen is a natural substrate for cellular attachment, growth and differentiation, and promotes cellular proliferation and differentiation. Once dermis reconstruction is done, the covering of the wound surface with both *in vitro* expanded epidermis and autologous split-skin transplants is significantly easier, and it has an improved chance of success (Z. RUSZCZAK [8]).

A wound dressing may be a single product, or may combine two or more layers of dressing materials consisting of a primary wound contact layer, and a secondary retention or abosptive layer which is not in direct contact with the wound. Biopolymeric membranes can be successfully used for guided tissue regeneration, while antibacterial materials have a significant reduce in subsequent complications or infections.

Chitosan has a wide range of utility, being a natural, biocompatible, biodegradable polymer with a higly efficient wound healing effect. Chitosan has a structural characteristic similar to glycosaminoglycans, which are widely distributed among various tissues and seems to mimic their functional behaviour. There is a vast amount of literature on the extracellular matrix macromolecules including collagen, glycosaminoglycans and glycoproteins, playing a crucial role in the building up of new tissue. It is proposed that wound healing is accelerated by the oligomers of degraded chitosan by tissue enzymes, and this material was found to be effective in regenerating the skin tissue of wound area. Commercially products are already available to the market, like INTEGRA™ Bilayer Matrix Wound Dressing, which is an advanced wound care device comprised of a porous matrix of cross-linked bovine tendon collagen and glycosaminoglycan, and a semi-permeable polysiloxane (silicone layer). The semi-permeable silicone membrane controls water vapor loss, provides a flexible adherent covering for the wound surface and adds increased tear strength to the device. Data in literature confirm that chitosan is a potential substitute for human novel wound dressings. A challenging area in the field of collagen-chitosan wound dressings is the use of electrospun nanofibrile membranes, which seems to potentially offer many advantages over conventional processes. On the account of the fact that electrospun nanofiber has a huge surface area and microporous structure, a nanofibrilar membrane could quickly start signaling pathway and attract fibroblast to the dermis layer, which can excrete important extracellular matrix components, such as collagen and several cytokines (e.g. growth factors and angiogenic factors), to repair damaged tissue (J. CHEN & al. [9], C.H. YAO & al.[10], H.P. FELGUEIRAS & M.T.P. AMORIM [11], S.B. QASIM & al. [12] X. GENG & al. [13]).

Another important aspect for new dressings consists in potential failures of the proper sterilization, leading to significant costs associated with nosocomial infections of patients and mortality issues (G.C.C. MENDES [14]). Any dressing used in the clinic should be sterile in accordance with

appropriate standards (E. MAZORA & M. ZILBERMAN [15]): EN ISO 11137-1:2015, EN ISO 11137-2:2015, EN ISO 11737-1:2006, EN ISO 11737-2:2009, EN ISO 13485:2016.

Sterile products can be obtained with methods using heat (dry heat or autoclaving) and the so-called "cold" processes, i.e. microbicidal gases (gas plasma of low-temperature hydrogen peroxide, low temperature peracetic acid gas, peroxide hydrogen vapors, ozone, chlorine dioxide) or high energy irradiation (M. GEIGER [16]).

In the case of collagen, sterilization with steam or dry heat is not applicable because can induce changes in protein chemistry and physical properties, affecting the absorption potential, mechanical strength or performance. An alternative to obtain sterile collagen products is ethylene oxide treatment, which is less common for drugs, but has a significant role in the sterilization of medical devices (R. PEACOCK [17]). Gamma irradiation is another established method of sterilization for collagen products (S.D. GORHAM [18]), and has been used for the preparation of bone grafts and human tendons (M. GEIGER [16]).

Since heat sterilization is not possible, sterilization with ethylene oxide is not applicable (not possible for liquid forms, and is difficult to validate for solids), and aseptic manufacturing is not an option given that the raw material is of animal origin (C. WIEGAND & al. [19]), according to E. MAZORA & M. ZILBERMAN [15], the irradiation is the most appropriate method of sterilization for wraps containing natural and sensitive polymers.

Although sterilization of collagen-based biomaterials is accomplished by low dose gamma irradiation (R. PARENTEAU-BAREIL & al. [20]), has been observed from previous studies that this method alters the molecular structure and decreases the mechanical and enzymatic resistance of the collagen scaffolds. Sterilization with ethylene oxide (ETO) or β-ray irradiation is less harmful than γ rays, but their applicability depends on the type of collagen products (E.M. NOAH [21]; W. FRIESS & M. SCHLAPP [22]). Immersion in a low peracetic acid concentration or formic acid are considered a potential collagen sterilizing agents (S.P. WILSHAW & al. [23], C.J. DOILLON & al. [24]). Immersion in ethanol with the combination of fungicides or/and antibiotics are techniques used in the laboratory for the sterilization of collagen scaffolds that have been physically crosslinked (L. MA & al [25]). However, according R. PARENTEAU-BAREIL & al. [20], no sterilization technique has proven optimal for sterilizing different forms of collagen. Investigating the effects of sterilization on the properties of collagen materials remains the best way to evaluate the performance of sterilized collagen.

Cross-linking is also required for biopolymers to control resistance to degradation and enhance mechanical integrity during sterilization (L.M. DELGADO & al. [26]). A bilayered collagen-chitosan wound dressing produced by using an electrospinning tehnique for the chitosan layer, is able to avoid molecular changes of collagen during sterilization, and the stability changes over time. More than that, chitosan layer has many advantages for wound healing, such as hemostasis, accelerating the tissue regeneration by enhancing the fibroblast collagen biosynthesis of (L. MA & al [25]), offering a much more promising effect of collagen dressings.

In this context, our aim was to improove the mechanical properties and antimicrobial activity of a collagen sponge matrices of medical device type, produced by a romanian manufacturer, by the development and sequential analysis of a bilayer chitosan-collagen (Col/CS) product.

2. Materials and methods

Sample Preparation

Chitosan solution was prepared at 2% (w/v) in 1 M acetic acid, at room temperature under magnetic stirring (100 rpm) for 3 days, to obtain homogeneous solution. The solution was centrifuged at 1500 RPM for 3 min prior to electrospinning, in order to remove air bubbles.

Collagen sponge production includes collagen extraction from animal tissues by solubilization with non-specific enzymes (5.2% w/w pepsin (250 units/mg) in HCl solution, at a pH value of 1.8), followed by (atello)collagen purification by diafiltration against purified water. Briefly, the process had the subsequent steps: raw material (bovine tendons) mechanical processing; physico-chemical individualization of quasi-native collagen fibers by (i) peptization of collagenic tissue and (ii) solubilization of globular and denatured protein fractions; isolation of colloidal suspensions by successive filtration steps; non-stoichiometric neutralization of the mixture and advanced purification of the (atello)collagen dispersions by diafiltration (10 kDa MWCO membranes); freeze-drying of the (atello)collagen dispersions to obtain porous solid substrates. The porous substrate were subsequently laminated in order to obtain Col structures (colapsed porous structures as substrates for the chitosan electrospun solution).

Electrospinning

The prepared CS solution was placed in a 50 mL plastic syringe fitted with a 21-gauge stainless steel needle. The syringe pump delivered solutions at 7 mL/h flow rate, and electrospun with an applied voltage of about 20 kV between the electrodes, using a Tong Li Tech electrospinnig equipment. The CS fibrils were deposited on a rotating collector (140 RPM) of 10 cm diameter and 30 cm length, covered with a Col structure of 10x20x0.2 cm (lxLxh) kept at 15 cm away from at the tip of the needle. The experiments were carried out using a heat output of 0.500 W, with relative humidity ranging between 50% and 60%. The produced nanofibers matrices were left in ambient conditions to evaporate the excess of acetic acid and water prior to further analyses.

Material appearance and physical characteristics

Appearance: The visual inspection was carried out for material appearance assessment. Also, the overview of a bilayer product Col/CS as cross-section was observed by using a ZEISS Axio Imager 2 microscopy platform, and ZEN imaging software, used for length measurements, in order to establish the chitosan electrospun film thickness.

Determination of Swelling Ratio and hydrolytic loss: In order to induce swelling, Col/CS and Col matrices were used, sampled as 5 mm diameter discs. The pre-weighed samples (W_0) of Col/CS and Col were immersed into deionized water at room temperature for 10 min. The samples were collected and the excess water was removed with filter paper, then were re-weighed (W_1). Swelling ratio (ε) was calculated as follows:

$$\varepsilon(\%) = \frac{(W1 - W0)}{W0} x100$$

Hydrolytic loss of Col/CS and Col matrices was evaluated by immersing them in deionized water for 10 min, at 37 °C. Degradation was appreciated by visual inspection.

Enzymatic lysis: To determine the time required for complete digestion of the bilayer product, in the presence of pepsin, as a proteolytic enzyme, it was weighed as samples of 45 -50 mg, and then wetted with pure water. After removing the excess water, 100 mL of 1% pepsin in 0.1 N HCl, at 37° C, were

added. The pepsin used has an enzymatic activity of 1.0 - 1.17 IU/mg. The mixture was kept in water bath at 37 °C, and checked every 2-3 minutes. The time required for pepsine digestion of the sample was determined when the product lost the integrity and structure, observing only light fibers in the solution. Col/CS and Col matrix as pozitive control were used, sampled as 5 mm diameter discs.

Col/CS bilayer morphology: The SEM analyses of the Col and Col/CS samples were performed using a FEI microscope. The samples were fixed with a double tape on a conductive support of aluminium. Afterwards, it was covered with a thin gold layer using sputtering for 60 sec. For the SEM measurements, the following parameters were used: high voltage 10 kV, working distance 10 mm, and a ETD detector for the detection of the secondary electrons.

The AFM analysis of the Col and Col/CS samples were performed using a AFM 5500 from Agilent Technology. The AFM measurements were performed in ambient atmosphere (23ºC, 35% relative humidity) in contact mode, using a silicon cantilever with elastic constant of 0.08 N/m, and a resonant frequency of 17 kHz. For the processing of the AFM images, the programe Gwyddion was used to evaluate the morphological parameters (the average square roughness, RMS, $RMS = R_q = \left(\sum_{i=1}^{N}\left[\frac{(h_i-\bar{h})^2}{N}\right]\right)^{1/2}$).

ATR-FTIR analysis: Fourier Transform Infrared (FT-IR) spectroscopy is a valuable tool for characterizing and identifying compounds or functional groups. FTIR spectra of CS and Col/CS were recorded in triplicate, at room temperature, using the FTIR Cary 630 spectrometer from Agilent Technologies, in ATR mode, in the range of 4000 to 650 cm^{-1}, with a spectral resolution of 2 cm^{-1} and 200 scans.

Bioburden determination

As stated by the standards for medical devices (ISO 11737 family), the appropriate method should be selected for bioburden determination, in order to ensure: removal of the viable microorganisms from the sample, culturing, and enumeration of the microorganisms. In this respect, we followed the pharmacopoeial rules for sterilization by using ionising radiation sterilisation, i.e. 25 kGy, in order to obtain a sterility assurance level (SAL) of 10^{-6}. The sterile samples were used for establishing the factor for removal rate of the viable microorganisms from the samples. Two standardized strains were used for the tests, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739. The product used for testing was defined as SIP (sample item portion) of 1 cm^2 surface. Vortex mixing method was chosen for viable microorganisms removal from the SIP. Inocula adjusted according to McFarland Standard 0.5 were obtained from 18-24 hours cultures of *S. aureus* and *E. coli* developed on solid media. The sterile SIPs were immersed in 5 mL of suspension, maintained for 10 min for incubation at ambient temperature, and recovered on a sterile bottle. Excess inoculum was removed with a sterile filter paper, and SIP immersed in 5 mL of Eugon broth. Each tube was vortexed for 10 sec / min for 5 min at 100 RPM. Each sample was left for 10 minutes at rest, then 550 µL of supernatant were harvested and serial decimal diluted. 1 mL of the 10^{-6} and 10^{-7} dilutions were evenly plated. Positive control (decimal serial dilution inoculum) and negative (AFS, eugon broth and uncontaminated SIP) were used. The recovery factor (RF) was calculated as

$$RF = \frac{UFC\ pos\ control}{UFC\ SIP}$$

Antibacterial activity assay

Qualitative screening for the antimicrobial activity of the Col/CS product (disks of 5 mm diameter) was made by using an adapted diffusimetric method. The antimicrobial qualitative screening was carried out against standardized microbial strains recomanded by the European Pharmacopoeia (8^{th} ed.) microbiological quality assessment for topical drug formulations: *S. aureus* ATCC 6538, *E. coli* ATCC 8739. The bacterial inocula were made of 18-24 hours cultures grown on solid media, and adjusted to a density of 1.5 x 10^8 CFU/mL, using the 0.5 McFarland standard. The Col/CS samples were placed on the plates evenly spreaded with bacterial inoculum. Both Col and CS from the Col/CS product were placed in contact with the inoculated plate. The microbial sensitivity was expressed as the diameter of the growth inhibition zones.

3. Results and Discussions

The materials design and morphology must be controlled in accordance with the purpose of their use. The electrospun nanofiber has played a pivotal role in the area of biomaterials, especially in the biomedical field, by menas of biocompatible and biodegradable (natural or synthetic) polymers, in order to obtain products with high surface area and superior mechanical properties. The electrospun biomaterials structures are related to the electrospinning parameters, i.e. (i) the applied electric field, distance between the needle and collector, flow rate, needle diameter; (ii) the solution parameters, including the type of solvent, polymer concentration, viscosity and solution conductivity; (iii) relative humidity and temperature of the air inside the electrospinning cabinet.

A plethora of materials structures were developed by using collagen and chitosan (A. SIONKOWSKA & al. [29]; J.P. CHENA & al. [30]; B. KACZMAREK & al. [31]), as simple ~~and~~ or combined matrices, some of them being available in marketable form. Our purpose was to improove the mechanical and antimicrobial properties of a collagen sponge manufactured by a romanian producer of medical devices. The description, embracing colour and physical form is an important test for pharmaceutical materials, and could be successfully adopted as a descriptor for the developed biomaterial. The term “white or almost white” was used for quality control purpose of the bilayer material, Col/CS.

The electrospinning technique was chosen for obtaining a bilayer product, with electrospun chitosan and a laminated freeze-dried collagen support. A thin film of about 9.5 μm of the chitosan has been deposited (**Fig. 1**).

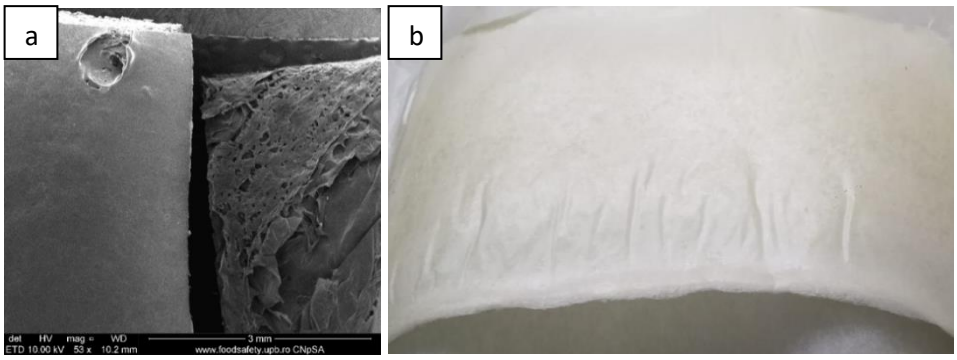

Fig. 1. Overview of the bilayer product Col/Cs. a. SEM image, b. visual inspection aspect.

Swelling ratio (ε) was calculated for the Col/CS product, and compared with the Col support matrix as positive control. Differrent swelling characteristics have been assessed for the support matrix, i.e. ε Col = 83.7%, and ε Col/CS = 43.5% for the Col/ CS product, presented as average of 3 separate determinations by using 5 discs of 5 mm diameter. A fractional hydration factor was calculated as:

$$FH = \frac{\varepsilon\ \mathrm{Col} - \varepsilon\ \mathrm{Col/CS}}{\varepsilon\ \mathrm{Col}} = 0.48$$

A certain humidity is essential for healing moist wounds, toghether with the aboundance of wound exudate which raises severe problems in clinical practice. Having in view the exudates qualitative and quantitative influence on the healing phase of specific wounds, particular products could be developed for lightly to heavily exuding wounds (V. ANDREU & al. [32]). In this respect, the FH parameter could be used as quality control for the developed product, in preserving the collagen matrix properties, including the absorbance capacity.

The major drawbacks associated with the use of protein based-polymers are their low mechanical stiffness and rapid degradation rate *in vivo* (V. ANDREU & al. [32]). A plethora of methods were used for increasing the biological stability, i.e. crosslinking through physical or chemical methods. The biological stability of the Col/CS and Col support matrices was evaluated by exposing them to the selective endopeptidase pepsin, to assess degradation times and calculate a comparative rate of degradation. The average of 3 separate determinations by using 5 discs of 5 mm diameter, revealed degradation time of 12.5 min for the Col/CS bilayer product, and 4.3 min for the pozitive control Col. The comparative rate of degradation was 1.98, revealing a factor of about 2 in resistance increse for the bilayer product, Col/CS.

Fourier-transform infrared (FT-IR) spectroscopy is based upon the absorption of IR radiation by vibrational transition in covalent bonds of the biomolecules. The IR absorbance provides information about the sample contents depending on their structure, the molecular bounds and their environment (K. BELBACHIR & al. [27]).

FT-IR spectra of Col, CS and Col/CS are presented in **Fig. 2**. In the case of Col spectra, four amide bands can be observed at the wavelengths of 3301 cm^{-1}, 3074 cm^{-1}, 1630 cm^{-1} and 1542 cm^{-1}. The Amide I band (1630 cm^{-1}) originate from the ν (C = O) coupled to the δ (N – H) absorption. The Amide II band (1542 cm^{-1}) is given by the δ (N – H) coupled with the ν (C – N) absorption. The other two Amides are from the δ (N – H) group, of medium to week intensity, appear at 3301 cm^{-1} and 3074 cm^{-1}, respectively. In the spectra of CS, the ν (OH) group was observed at 3305 cm^{-1} and the ν (C – H) bond at 2911 cm^{-1}. The absorbance peaks at 1650 cm^{-1}, 1534 cm^{-1}, 1400 cm^{-1} and 1308 cm^{-1} are attributed to the presence of ν (C = O), δ (N – H), ν (C – H) and ν (OH) characteristic to the chitosan structure. After the deposition, the peaks of Col/CS have modified values of absorbance. Since the intensity of $–NH_2$ band in collagen molecule is stronger than that of –NH–, the changes of Amide II bands indicate that the free $–NH_2$ group in collagen molecule were converted to –NH– groups (i.e. intermolecular links between chitosan and collagen or within collagen moleculars formed) (X.H. WANG & al. [28]).

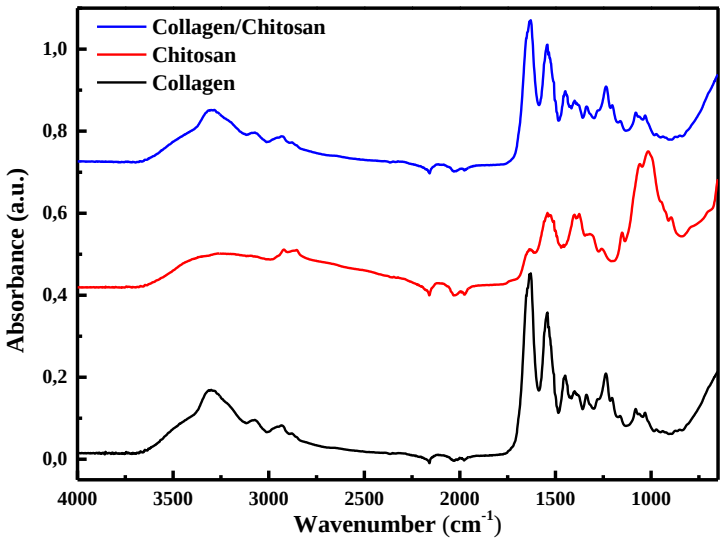

Fig. 2 FT-IR spectra of Col (black line), CS (red line) and Col/CS (blue line).

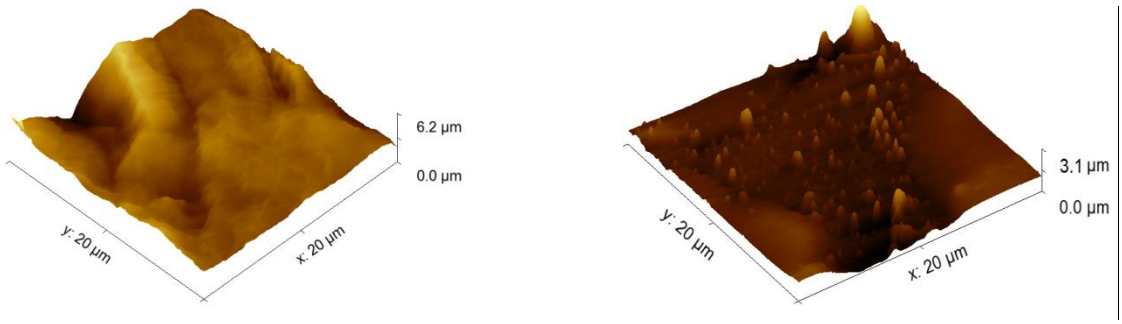

Fig. 3 AFM images of Col (left) and Col/CS (right) samples.

AFM images of the Col and Col/CS samples are presented in **Fig. 3**. From the processing of the AFM images we could evaluate the roughness morphology using a dedicated software. As expected the roughess of the Col sample (RMS of 620 nm) was much higher than the roughness of the Col/CS sample (RMS of 340 nm). The results showed a lot of beads disposed on the CS nanofibers surface. Further development would be necessary, in order to find the appropriate experimental conditions for obtaining the nanofibers layer without beads.

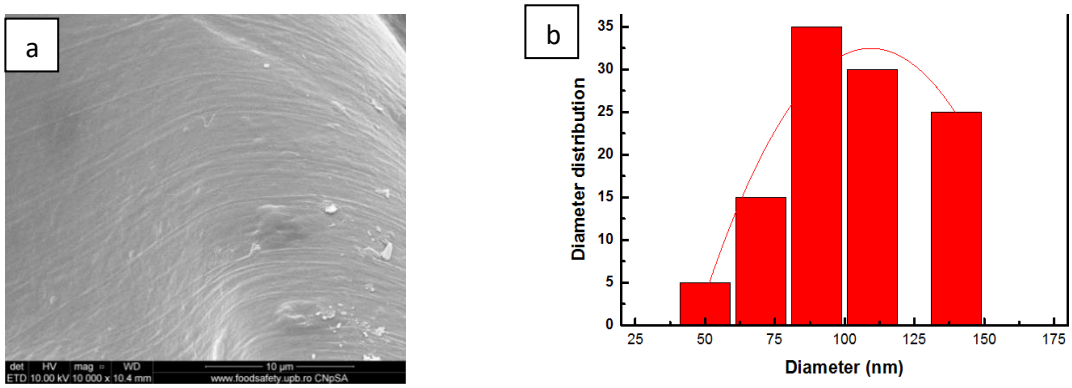

Fig. 4 SEM images and diameter distribution (b) of the Col/ CS matrix (CS layer view –a).

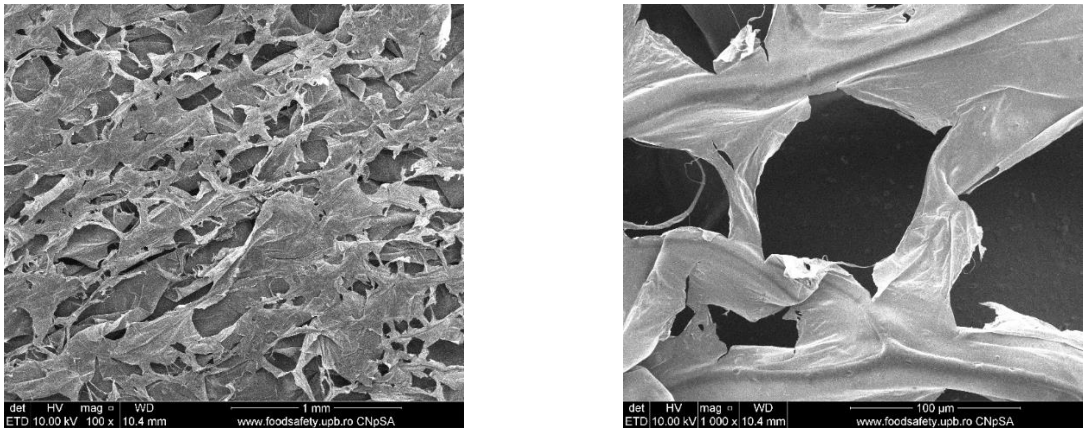

Fig. 5 SEM images and pore distribution of the Col support matrix.

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SEM images and diameter distribution of the Col and Col/CS samples are presented in **Fig. 4 and 5**. As could be seen in Figure 4, the results of the Col/ CS sample exhibits aligned nanofibres with the average diameter, d_{Col}, of 102 ± 2 nm. In **Fig. 5** we can observe that the Col support matrix exibits evenly distributed and interconnected pores.

The mandatory requirement for medical devices is the efficiency of microorganism recovery techniques in wound dressings. After sterilization of Col/CS matrix, the correction factor for recovery efficiency was determined, in accordance with European Pharmacopoeia, 8th ed. Five distinct stages are involved: sample selection, removal of microorganisms from sample, transfer of microorganisms to recovery conditions, enumeration of microorganisms with specific characteristics, and interpretation of data, involving application of appropriate correction factors determined during validation studies. In our study, the recovery efficiency of *S. aureus* and *E. coli* from Col/CS matrix was 36% and 52%, respectively. It was assumed that the results confirm the presence of the antimicrobial effect of the product. The recovery efficiency for *E. col*i was better than that for *S. aureus*, probably due to the difference in cell walls between Gram-positive and Gram negative bacteria. This result was also confirmed by the qualitative determination of antimicrobial activity (average diameter of the inhibition zone for the discs tested against standardized microbial strains was 9 mm for *S. aureus,* and 8 mm for *E. coli*).

Non-healing wounds has a worldwide negative impact, for example in the US, the chronic nonhealing wounds affected nearly 15% of Medicare beneficiaries (8.2 million). The annual cost was conservatively estimated at $28 billion when the wound was the primary diagnosis on the claim. When the analysis included wounds as a secondary diagnosis, the cost for wounds was conservatively estimated at $31.7 billion (S.R. NUSSBAUM & al. [33]). At the site the bacterial cells produce and secrete a variety of enzymes and toxins. A bacterial population of 10^5 colony forming units (CFU) per g or cm^2 indicates an infected wound. Such a bacterial load can be reduced by using an antimicrobial dressing (J. FONG & F. WOOD [34]). In this context, the proved antimicrobial activity and mechanical characteristics recommend the developed Col/ CS product as a practical solution for chronic wounds management.

4. Conclusions

In this study, certain steps were proposed for the development of a collagen–chitosan bilayer product, appropriate for biomedical use. The sequential analysis including product appearance and physical characteristics, morphology, and antimicrobial properties are the premises for laboratory validation of the new product. Also, important determinations for sterilization validation have been covered, i.e. sample selection, removal of microorganisms from sample, transfer of microorganisms to recovery conditions, enumeration of microorganisms with specific characteristics, allowing the calculation of the correction factor for the product contamination analysis. However, it is still necessary to implement further improvements of the electrospinning parameters of the chitosan layer.

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