Drug loaded chitosan wound dressings: choosing the best sterilization method

Eleni Nydrioti

Supervisor: Doctor Ana Paula Valagão Amadeu do Serro

Supervisor: Doctor Benilde de Jesus Vieira Saramago

Abstract

Introduction: Wounds may be caused by surgery, trauma, or as a result of diseases such as diabetes. The disruption of the skin/internal tissues and the contact with the external environment may lead to microbial infection. Wound dressings are capable of providing a protection barrier and can accelerate the wound healing process. Chitosan is one of the most promising materials for wound dressings, due to its good biocompatibility, low toxicity, hemostatic properties, antibacterial activity and biodegradability. The main goal of this work is to produce efficient and safe chitosan based hydrogels and choose the best sterilization method.
Experimental: Three different chitosan based materials were produced starting from BioceraMed formulations, AbsorKi®, HidroKi® and HemoKi. The latter was modified by addition of genipin, a cross-linking agent. The dressings were individually loaded with two different drugs, Polyhexamethylene biguanide (PHMB, also called polyhexanide) and chlorhexidine diacetate (CHX), by soaking in solutions containing each drug. The physical properties (swelling, mechanical properties, wettability and morphology) of the materials were studied before and after sterilization. The methods used for sterilization were: steam and pressure, high hydrostatic pressure (HHP) and gamma radiation. Chorioallantoic membrane (HET-CAM) tests were done to non-sterilized dressings to study potential irritation of the skin. The antibacterial activity of the drugs was assessed by measuring the optical density of an incubation solution containing drug loaded WD and bacterial suspension. *In vitro* drug release studies were performed with a Franz diffusion cell system, combined with UV-Vis absorption spectroscopy for drug quantification. Since HHP is considered a relatively novel sterilization method its efficiency was evaluated by sterility tests.
Results: The lyophilized dressings present a porous structure and a high swelling before and after sterilization. HPP increased the swelling of AbsorKi®, but all methods decreased the swelling of HemoKi. The drug release profiles indicated that the concentrations of PHMB and CHX increased in a controlled way after sterilization within the first 8h for all the methods. The only exception was HidroKi® loaded with PHMB. HHP improved the mechanical properties of both dry dressings while for HidroKi® a small improvement in the properties was observed after steam and pressure sterilization. The HET-CAM tests suggested that the produced materials do not lead to irritation and loaded with drugs can be very effective against bacteria.
Discussion and Conclusions: HHP revealed an efficient method to ensure the materials sterilization making the drug loaded wound dressings potentially efficient devices for the absorption of exudate from the wound bed. The combination of chitosan, a natural antibacterial agent, with the studied disinfectant and antiseptic drugs may lead to promising materials to be used as drug delivery platforms.

Keywords: wound dressings, chitosan, drug delivery, HHP, steam and pressure, gamma radiation

1. Introduction

Wound healing is a very complex process and one of the major risks that can occur is contamination of the wound in any of the four stages of healing [1]. A wound dressing (WD) is a sterile pad designed to be in contact with the wound in order to promote healing and protect the wound from further harm [2]. Over the years several WD have been on the market, such as semi-permeable film or foam dressings, hydrogels, hydrocolloid, alginate, bioactive etc [3].

Hydrogel-based WD are one of the most promising materials in wound care due to their properties, such as high capacity of fluid sorption, providing moisture to the wound etc

[4]. Chitosan is a natural copolymer widely used in medicine due to its biocompatibility, non-toxic, antimicrobial and haemostatic properties [5], [6]. Chitosan WD incorporating antibiotic or antiseptic agents are used for local drug delivery and effective treatment against infections [7].

Sterilization of hydrogels is particuarly challenging for the biomedical industry due to the sensitivity of this material to common sterilizing agents such as heat and irradiation [8], [9]. The existance of water in the hydrogel structure can contribute the breakdown of chemical bonds and initiate possible alterations in the material properties, affecting their safe performance [10]. The outcome of sterilization proccesses is even more demanding for combinatory medical products including drug delivery systems, in which attention must be paid to material integrity and factors related drug degradation/stability and changes in the release profile [11-14].

In this work was made an effort to develop chitosan based WD and evaluate the effects of several sterilization techniques on these materials. Two conventional and widely used methods, namely steam and pressure and gamma radiation, and one relatively new method (HHP) were studied.

2.1 Materials

Acetic acid, 2% (v/v) from Sigma-Aldrich®; Lactic acid, 2% (v/v) from Sigma-Aldrich®; Chitosan, Chitopharm L, 620 kDa – 79.4% D.D. Batch: UPBH3114PR from BASF was supplied by BioceraMed (Portugal); Ammonium hydroxide 2.5% (v/v) from Panreac; Chlorhexidine diacetate from Santa Cruz Biotechnology, Inc.; Polyhexamethylene biguanide from Sigma-Aldrich®; Genipin from CarboSynth; Sodium Chloride from Fluka Analytical; Potassium Chloride from Sigma-Aldrich®; Sodium dihydrogen phosphate monohydrate from Merck; Sodium Hydrogen Carbonate from PanReac AppliChem; Lysozyme from Fisher BioReagents; Mueller-Hinton Broth from Oxoid; Thioglycollate Liquid Medium from PanReac AppliChem; CASO Broth from Sigma-Aldrich®.

2.2 Preparation of chitosan based wound dressings

The production of AbsorKi® (dry dressing) and HidroKi® (hydrated dressing) was based strictly on formulations provided by BioceraMed while HemoKi (dry dressing) was further developed by adding genipin, a natural cross-linking agent. The first step in the synthesis of AbsorKi® was to fully dissolve chitosan (3% m/m) in an acetic acid solution (2% v/v). Then the solution was placed in Petri dishes and was put a freezer at -80ºC overnight. Afterwards, the plates were lyophilized for 24 hours. For the coagulation of the hydrogels was used an ammonia solution (2.5% v/v) where the lyophilized samples were immersed into for 10 min. Before the final lyophilization the samples were washed with DD water to remove any remaining ammonia.

To produce HemoKi, chitosan (3% m/m) was added in an acetic acid solution (2% v/v) and stirred until it was dissolved completely. Then genipin, which was previously dissolved in DD water, was added in the chitosan solution (1mg/mL) and stirred until it became homogeneous. For the cross-linking of the hydrogel, the chitosan-genipin solution was placed in the oven for 15 hours at 26ºC. Finally, the samples were lyophilized in the aforementioned conditions.

HidroKi® was prepared by dissolving chitosan (3% m/m) in a solution of lactic acid in DD water (2% v/v) and then was deposited in silicone moulds. In this case, ammonia vapour was used in the coagulation step. A solution of ammonia (2.5% v/v) was prepared and placed in a Petri dish in the bottom of a glass chamber to create ammonia vapour. The plates containing the chitosan solution were suspended on top of this chamber which was airtight sealed. After 24 hours the samples were removed from the chamber and were washed with DD water three times and were left to dry for 30min to remove any remaining ammonia. Finally, they were stored inside airtight sealed bags with 3mL of DD water to guarantee that the samples would remain hydrated. The final WD are presented in Fig. 1.

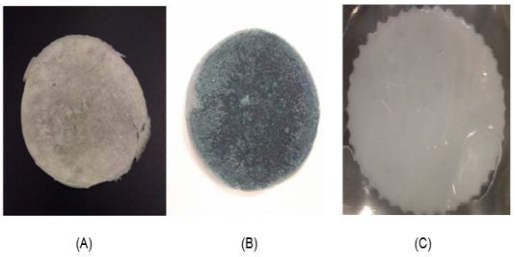

Figure 1:Photos of the final form of the dry dressings. (A) AbsorKi® and (B) HemoKi with the characteristic blue colour on the right and HidroKi® (C).

2.3 Drug loading and drug release procedures

Samples of the three materials were drug-loaded by soaking, at 36 °C, in 5 mL of drug solutions: 0.5 mg/mL of PHMB for 24 h, 180 rpm and 5 mg/mL of CHX for 24 h, 180 rpm and 5 mg/mL. Polyhexanide was dissolved in pseudo extracellular fluid solution (PECF) while chlorhexidine was dissolved in DD water due to its low solubility in saline solution.

Drug release experiments for all dressings, before and after sterilization, were performed using a Franz cell diffusion system. Six receptor cells were filled with preheated PECF solution (6.5 mL) and were placed into the Franz cell system. The drug loaded samples were fixed between the donor and receptor compartment. The diffused drug was magnetically stirred constantly. Aliquots of 200 µL were removed at predetermined times to measure the drug concentration in solution, being replaced by the equal volumes

of fresh preheated PECF solution. Six experiments were carried out for each system. Due to an error occurred during the coagulation of AbsorKi® it was impossible to obtain drug loaded samples (CHX) for steam and pressure and gamma radiation. Therefore, this dressing was submitted only to HHP sterilization.

The concentration of released drugs in the collected solutions was determined using a UV-Vis spectrophotometer (Multiskan GO, Thermo Scientific), at λ=235nm for PHMB and λ=255nm for CHX.

2.4 Sterilization

To study the effects of sterilization on the dressings three different sterilization methods were used: steam and pressure, high hydrostatic pressure (HHP) and gamma radiation and. The dry dressings (AbsorKi® and HemoKi) were sterilized by all methods and HidroKi® was sterilized only by steam and pressure and HHP. For all methods were sterilized blank and drug loaded samples.

2.4.1 Steam and pressure

In the steam and pressure sterilization, the parameters applied for all materials were: temperature at 121^{0}C, pressure of 1 bar and time period of 20 minutes. The blank dressings were sterilized in falcons: the dry ones were simply placed in tubes and the hydrated ones were incubated with 3mL of DD water. During the sterilization process HidroKi® samples were loading in drug solutions (5mL).

2.4.2 High hydrostatic pressure

For the HHP sterilization, AbsorKi® and HemoKi samples (blank or drug loaded) were sealed in bags from which the air was removed with vacuum. Blank HidroKi® samples were packaged with DD water to maintain their hydrated state throughout the sterilization process while the drug loaded samples were immersed in drug solutions (5mL). Prior to sterilization, water was heated in an external water bath and then it was poured in an insulation vessel alongside with the samples. Once the water temperature reached the 70ºC, the insulation vessel was placed in the machine. The software program used had the following settings: temperature at 70ºC, pressure at 600MPa and time duration of 10 minutes [15], [16].

2.4.3 Gamma radiation

Lyophilized blank and drug loaded AbsorKi® and HemoKi samples were irradiated with 25 kGy using a Cobalt-60 source with a dose rate of 5 KGy/h.

2.5 Physical Characterization

Blank and drug loaded samples were characterized with respect to some of their physical properties.

2.5.1 Swelling capacity

The swelling kinetics of the three different materials was assessed before and after sterilization. Before the assay starts, the hydrated samples were blotted gently with absorbent paper to remove the excess of water from their surface. All samples had a dimension of 8 mm diameter. They were pre-weighted and afterwards were immersed in DD water and incubated for 48 hours at room temperature. At pre-determined time intervals, the samples were retrieved, carefully blotted with absorbent paper, weighted in the balance and immersed again in DD water. This process was repeated until the sample's weight reached equilibrium and the swelling stagnated. The weight of the samples was registered each hour for the first 8 hours and, after that, at 24 hours and 48 hours. The swelling capacity was calculated using the equation:

$$(\%)SC = \frac{W_t - W_0}{W_0} \times 100$$

where W_t is the weight of the hydrogel at each time point measured and W_0 is the initial weight.

2.5.2 Wettability

The wettability was determined by measuring the contact angles of captive air bubbles placed with the aid of an inverted needle underneath the sample-substrate system which was immersed in water. A video camera (jAi CV-A50, Spain) connected to a microscope Wild M3Z (Leica Microsystems, Germany) was used to acquire the images which were analysed afterwards with ADSA software (Axisymmetric Drop Shape Analysis, Applied Surface Thermodynamics Research Associates, Toronto, Canada). Wettability studies were conducted only for HidroKi® since the extremely porous surface of the lyophilized samples made the measurement impossible.

2.5.3 Mechanical properties

To evaluate the mechanical properties of the dressings were carried out tensile tests for the lyophilized dressings and compression tests for the hydrated before and after sterilization. Tensile strength (TS), %elongation (%E), Young's modulus (YM), breaking strength (BS) of AbsorKi® and HemoKi were calculated after tensile tests. The compression strength (CS) and Young's modulus were measured after conducting compression tests. All the values were determined by using the TA.XT Express Texture Analyzer (Stable Micro Systems).

2.5.4 Morphology

The morphology of the samples was analysed using a Field Emission Gun (FEG) SEM JEOL JSM-7001F SEM. Before the SEM analysis, the surfaces were coated with gold (Au) and palladium (Pd). For the tests, samples were cut in small discs (5 mm diameter), placed in the -80^{0}C freezer for 1 day and lyophilized overnight. SEM images were obtained under the following magnifications: x40, x200 and x1000.

2.5.4 Irritation (HET-CAM test)

Fertilized hen's eggs were incubated (Intelligent Incubator 56S) at 37 ± 0.5°C with 60 ± 3% RH for 8 days. During this period the eggs were manually rotated 180° three times a day and on the 9th day they were cut using a rotary saw (Dremel 300, Breda). Upon the removal of the cut part of the shell, with the aid of a scalpel, the inner membrane was moistened with a 0.9% NaCl solution and the eggs were incubated again for 30 minutes. Then the inner membrane was removed carefully and the chorioallantoic membrane (CAM) was exposed. Triplicates of non- sterilized AbsorKi®, HemoKi and HidroKi® samples (discs of 8 mm diameter) were placed directly on the CAM, and remained there for 5 minutes. NaCl (0.9%) and NaOH (1 M) solutions (300μL) were used as negative and positive controls, respectively. The progress of the process was observed within the time that was mentioned above and the occurrence of possible lysis, haemorrhage and coagulation of the blood vessels on the CAM was documented.

2.6 Microbiological tests

2.6.1 Sterility testing

Since HHP sterilization is a relatively recent method, its reliability needs to be evaluated. Therefore, sterility tests were carried out only for the dressings subjected to this method. The conditions applied during steam and pressure sterilization and sterilization by gamma radiation ensure that the dressings are sterile.

For the sterility tests two mediums were prepared: Thioglycolate Medium (TIO) used for observation of potential bacterial growth and Caso-Broth (Tryptone-Soya) for fungal growth. The Thioglycolate Medium was incubated at 30ºC for 14 days while the Caso-Broth was incubated at 25ºC for the same period of time. Triplicates of the different type of dressings (discs of 8 mm diameter) were immersed in 50 mL of the mediums contained in Schott flasks, under aseptic conditions, and then they were placed in the oven under the appropriate temperature depending on each microorganism. The evaluation of the sterility was done by visual inspection of turbidity, which indicates the presence of microorganisms in the medium due to contaminated samples.

2.6.2 Antibacterial activity

The antibacterial activity of the dressings, which presented the best release profiles and physical properties, was tested against *P. Aeruginosa* (ATCC 15662) and *S. Aureus* (ATCC 25923) by turbidimetry in Mueller-Hinton Broth (MHB, BD Quilaban. The bacterial suspension was prepared by removing colonies from the respective bacteria and by adding them to a 0.9% NaCl sterile solution to achieve a turbidity of 0.5 McFarland for *P. Aeruginosa* and 1 McFarland for *S. Aureus*. Afterwards it was added to the broth.

Triplicates of each sample (discs of 5 mm diameter) were placed individually in a 24-well plate. Then, 10 μL of bacterial suspension and 500 μL of MHB were deposited on each well. Three of these wells corresponded to the positive control. Only the wells corresponding to the negative control did not contain any bacteria. Then the plates were incubated in the oven at 37ºC under stirring at 100rpm for 24 hours.

After 24h, 200 μL of homogenized solution were extracted and placed individually in a 96-well plate. The optical density was measured using a spectrophotometer (Platos R 496 Microplate Reader) by reading the wavelength at 630 nm. All the procedures mentioned previously were carried out in a laminar flow chamber to guarantee aseptic conditions. The experiment was performed for both drugs PHMB and CHX.

3. Results

3.1 Evaluation of physical properties

The evaluation of the effect of different sterilization methods on the materials, was done by comparing the obtained results from sterilized and non-sterilizes samples.

3.1.1 Swelling capacity results

AbsorKi® sterilized by HHP exhibited the highest swelling (2296.5%) among all the other sterilized samples. Steam and pressure and gamma radiation samples increased the swelling capacity up to 692.6% and 1467.2%, respectively, relatively to the non-sterilized samples (531.3%). The swelling profiles of AbsorKi® are represented graphically in Fig. 2.

All sterilization methods led to a decrease in the swelling capacity of HemoKi. The lowest swelling (310.9%) resulted from steam and pressure sterilization. The swelling ratio of the samples sterilized by HHP increased (1195.3%) compared to the steam and pressure sterilization but it is still

lower compared to the non-sterilized samples. Finally, the sterilization by gamma radiation led to highest swelling capacity (1467.2%) compared to the other two sterilization methods. The swelling capacities of HemoKi are demonstrated in Fig. 3.

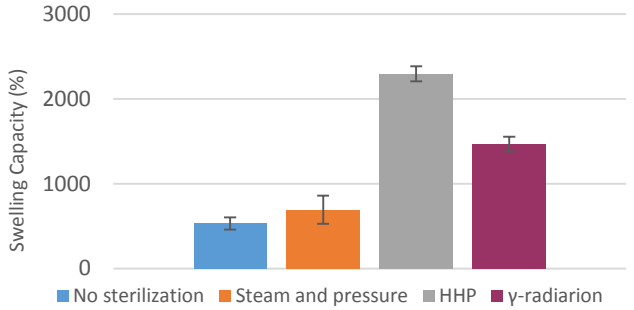

Figure 2: Swelling profile of AbsorKi® (with and without sterilization) in DD water for 48 hours at room temperature. The error bars are the maximum ± standard deviations (n = 4).

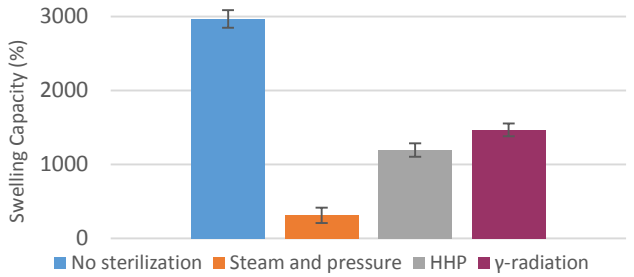

Figure 3: Swelling profile of HemoKi (with and without sterilization) in DD water for 48 hours at room temperature. The error bars are the maximum ± standard deviations (n = 4).

In Fig. 4 is presented the swelling of non-sterilized HidroKi® and sterilized by steam and pressure and by HHP. It is obvious that both methods increased the ability of HidroKi® to absorb water compared to the non-sterilized material (8.2%). Steam and pressure sterilization caused the most significant change leading a swelling capacity of 35%, while HHP led to 28.1%.

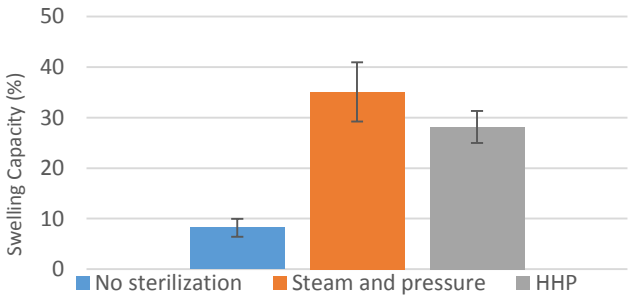

Figure 4: Swelling profiles of HidroKi® prior and upon sterilization in DD water for 48 hours at room temperature. The error bars are the maximum ± standard deviations (n = 4).

3.1.2 Wettability results

Sterilization by HHP caused an increase of approximately 5º in the contact angle compared to non-sterilized samples (42º ± 4º vs 38º ± 6º), thus decreasing the wettability. Steam and pressure sterilization had a small effect on the wettability of HidroKi®, relatively to non-sterilized samples, since the difference between the contact angles before and after sterilization is not significant.

3.1.3 Mechanical properties results

The experimental results of AbsorKi® for the different tested conditions are listed in Table 1. The values of YM indicate that the irradiation made the material more rigid (low tensile strain) and the BS was reduced to the half compared to non-sterilized samples. On the other hand, sterilization by HHP resulted to a significantly higher TS value is, which makes the dressing capable of remaining intact for longer time while it is being elongated. The YM is moderate but higher than the values obtained with the other samples. Regarding the findings for the effect of steam and pressure on AbsorKi®, it can be inferred that there was a slight change in the properties of the sterilized samples compared to the non-sterilized. The material lost a part of its elasticity (lower YM) and the capacity to resist to load (lower TS).

Table 1: Mechanical properties of AbsorKi® obtained from tensile test.

Sterilization method	Tensile strength, MPa ± SD	Elongation at break, % ± SD	Young's Modulus, MPa ± SD	Breaking strength, MPa ± SD
No sterilization	0.5 ± 0.3	2 ± 2	0.6 ± 0.1	0.3 ± 0.2
Steam and pressure	0.27 ± 0.15	3 ± 2	0.09 ± 0.04	0.2 ± 0.2
HHP	3 ± 2	16 ± 4	1.2 ± 0.4	1.8 ± 0.4
Gamma radiation	0.19 ± 0.05	2.1 ± 1.2	0.03 ± 0.01	0.15 ± 0.05

The results from the mechanical tests of HemoKi which are presented in Table 2. Sterilization by gamma radiation has a negative effect on the material by lowering its resilience and resistance to strain. Sterilization by steam and pressure made the material more brittle and stiff (lower TS and YM values). The only sterilization method that improved in general the properties of HemoKi samples is HHP. According to the value of TS it is obvious that the material became more resistant and can withstand more stress until it raptures completely. The E% increased significantly implying that the shape of the material is retained during the application of force while the elasticity is also increased.

Table 2: Mechanical properties of HemoKi obtained from tensile test.

Sterilization method	Tensile strength, MPa ± SD	Elongation at break, % ± SD	Young's Modulus, MPa ± SD	Breaking strength, MPa ± SD
No sterilization	1.7 ± 0.8	10 ± 7	0.19 ± 0.04	1.2 ± 0.8
Steam and pressure	1.4 ± 0.7	20 ± 9	0.12 ± 0.07	1.4 ± 0.7
HHP	4.6 ± 0.9	39 ± 7	0.9 ± 0.2	3.9 ± 0.7
Gamma radiation	1.16 ± 0.02	14 ± 3	0.17 ± 0.02	1.1 ± 0.1

In Table 3 are listed the results from the compression tests for HidroKi®. From the YM and CS values obtained after sterilization by HHP and steam and pressure sterilization, it can be inferred that the material is not resilient and not resistant when force is applied. However, the obtained values after steam and pressure sterilization are slightly increased compared to the HHP values.

Table 3: Mechanical properties of HidroKi® obtained from compression test.

Sterilization method	Young's Modulus, MPa ± SD	Compression strength, MPa/mm ± SD
No sterilization	0.028 ± 0.004	0.27 ± 0.02
Steam and pressure	0.031 ± 0.005	0.32 ± 0.07
HHP	0.024 ± 0.004	0.26 ± 0.01

3.1.4 Morphology results

SEM images of all dressings before sterilization demonstrate that the lyophilization process allows obtaining matrices with a highly interconnected porosity (Fig. 4 A-B) and that the surface of the hydrated sample is very irregular with many well defined pores (Fig. 5C).

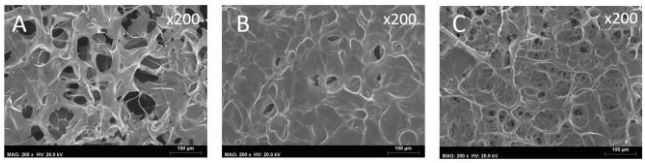

Figure 5: SEM images of AbsorKi® (A), HemoKi (B) and HidroKi® (C) before sterilization at magnification x200.

From the SEM images of dressings after steam and pressure sterilization can be seen that the surface of AbsorKi® (Fig. 6A) still has pores but is more flat compared to the non-sterilized. For HemoKi. The pores have almost been removed and the surface appears to be very smooth and dense (Fig. 5B).

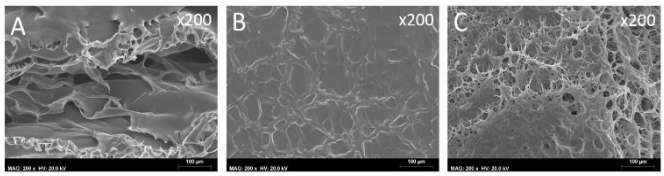

Figure 6: SEM images of AbsorKi® (A), HemoKi (B) and HidroKi® (C) after steam and pressure sterilization at magnification x200

The surface of HidroKi® became more rough and the pores are not well defined anymore (Fig. 6C).

HHP generated tremendous alterations on AbsorKi®, the surface is very compressed and smooth while there are no evident signs of pores (Fig. 7A) compared to the surface of non-sterilized material. The surface of HemoKi became denser and compressed uniformly but small pores still exist (Fig. 7B). Similar was the effect of HHP on HidroKi®. Its surface was compressed and the size of the pores decreased (Fig. 7C).

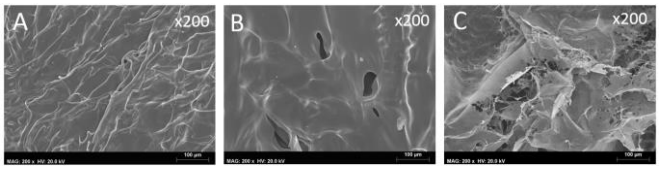

Figure 7: SEM images of AbsorKi® (A), HemoKi (B) and HidroKi® (C) after HHP sterilization at magnification x200.

Fig. 8A presents the effect of sterilization by gamma radiation on AbsorKi®. The surface is very rough and the pores seem to be enhanced compared to the other sterilization methods. Moreover, the surface of HemoKi is still smooth and dense in some parts but in others it became irregular and slightly rough. Unlike it happened after HHP, numerous small pores are still evident on the surface of the sample.

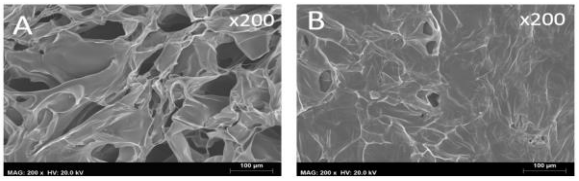

Figure 8: SEM images of AbsorKi® (A) and HemoKi (B) after gamma radiation sterilization with 25 kGy at magnification x200.

3.1.5 Irritation (HET-CAM test) results

Images of CAM (Fig. 9 A-C) after 5-minute exposure to non-sterilized samples of the three different materials did not show any visual lysis, haemorrhage or coagulation. On the other hand, Fig. 9E shows the response of CAM to the positive control (addition of NaOH) where haemorrhage and coagulation of the CAM was observed almost instantly and increased with time.

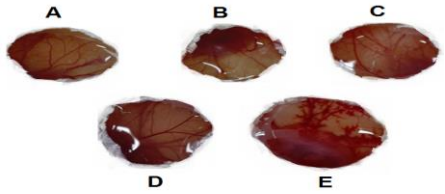

Figure 9: Chorioallantoic membrane images after 5-minute contact with not sterilized: AbsorKi® samples (A); HemoKi samples (B), HidroKi® samples (C); negative control (D) and positive control (+).

3.2 Evaluation of drug release profiles of WD

The PHMB release profiles for AbsorKi®, HemoKi and HidroKi® are shown in Figs. 10, 11 and 12, respectively. The AbsorKi® results show that all the release curves are similar. It is observed an initial burst in the first 8h and then a plateau is reached. The drug concentration after gamma radiation and HHP lower compared to steam and pressure and non-sterilized releases (Fig. 10).

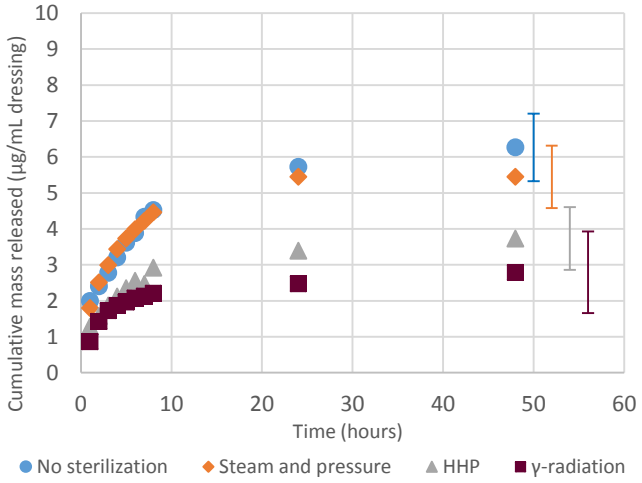

Figure 10: PHMB release profiles from AbsorKi® samples non-sterilized and sterilized by three different methods. The error bars are the maximum ± standard deviations (n = 6).

Similar behaviour can be observed from the release curves of HemoKi. The dressings release most of the drug within the 8h and then the release is sustained. For all the methods the released amount of PHMB is not very different (Fig. 11).

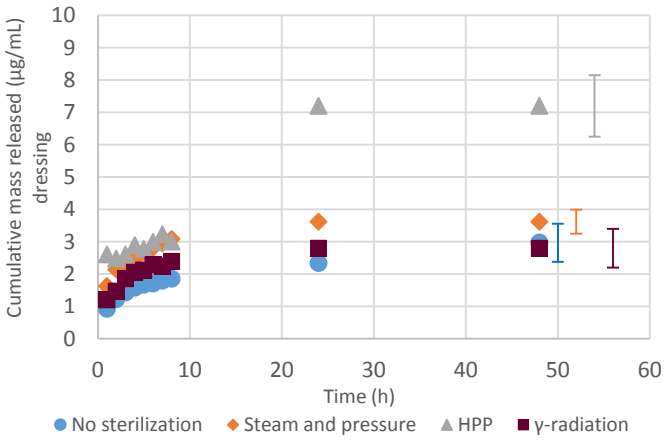

Figure 11: PHMB release profiles of HemoKi samples sterilized by the three different methods and not sterilized. The error bars are the maximum ± standard deviations (n = 6).

For HidroKi® can be seen that the release of the drug from the samples sterilized by steam and pressure and HHP is very low. In the first 8 hours the drug was released from both dressings but the amount is significantly low. After the 48 hours the releases from all dressings reached a plateau (Fig. 12).

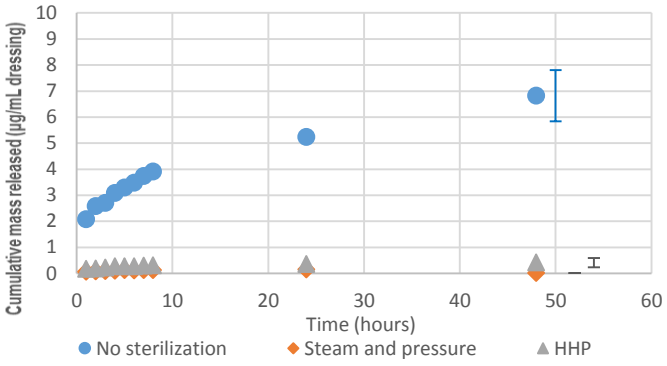

Figure 12: PHMB release profiles of HidroKi® non-sterilized samples and sterilized by the two different methods. The error bars are the maximum ± standard deviations (n = 6).

In Figs. 13, 14, 15 are presented the CHX release profiles of AbsorKi®, HemoKi and HidroKi®, respectively. In the first hours of release from AbsorKi® there is a burst and most of the drug is released after the 24 hours (Fig. 13).

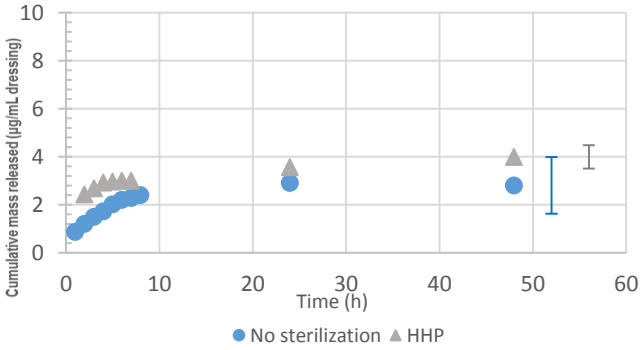

Figure 13: CHX release profiles of AbsorKi® samples sterilized by HHP and without sterilization. The error bars are the maximum ± standard deviations (n = 6).

HemoKi release profiles demonstrate that within the first 8 hours the release of CHX in the case of steam and pressure sterilization is slow and the total amount of drug increased slightly compared to the CHX release from samples sterilized by HHP and gamma radiation where the released amount is almost equal. The concentration of CHX released by non-sterilized samples has a similar release curve but the amount is lower relatively to the sterilized samples (Fig. 14).

It is possible to see that both HidroKi® sterilized samples yield similar release profiles with no significant difference between the released amount of CHX. In the first 8

hours the concentration of the released drug is low but remains sustained (Fig. 15).

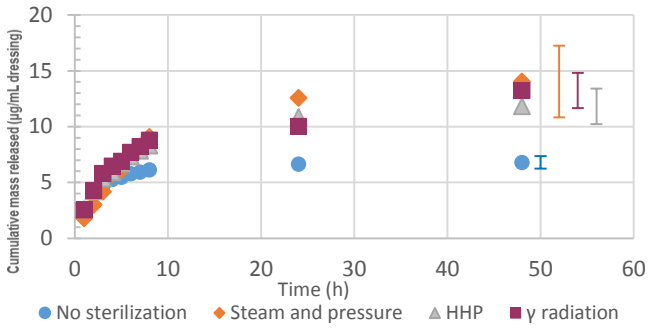

Figure 14: CHX release profiles of HemoKi samples non-sterilized and sterilized by the three different methods. The error bars are the maximum ± standard deviations (n = 6).

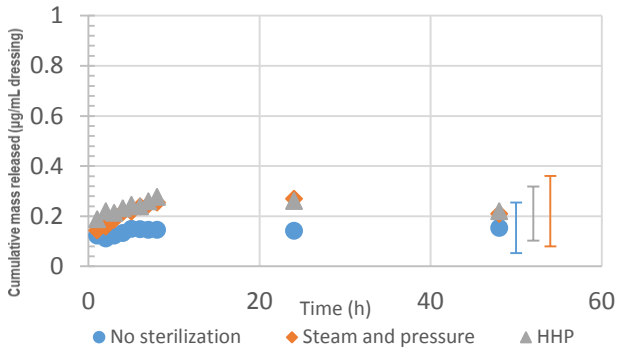

Figure 15: CHX release profiles of HidroKi® samples sterilized by the two different methods, steam and pressure and HHP sterilization. The error bars are the maximum ± standard deviations (n = 6).

3.3 Evaluation of microbiological tests

3.3.1 Sterility results

After the 14 days of incubation the dry samples did not show any turbidity in the medium. Therefore, both AbsorKi® and HemoKi were sterilized successfully by HHP. The hydrated samples showed some turbidity suggesting possible contamination. Extracted liquid of the turbid medium was spread on a Petri dish containing Mueller Hinton Agar. Afterwards the plate was incubated for 24 hours at 36°C. Bacterial colonies were formed on the plate verifying the contamination. Since the other two mediums with the HidroKi® samples were sterile, it was concluded that the bacterial colonies were formed due to cross contamination in the lab and it was not related to the sterilizing process.

3.3.1 Microbiological activity

The antibacterial activity of the drug loaded samples was assessed by measuring their optical density after being incubated individually with solution containing *S. Aureus* and *P. Aeruginosa*, respectively. The tested samples were loaded with PHMB and CHX and were sterilized with different methods. For the selection of each method not only the drug release profiles were considered but also results referring to the properties of the materials after being submitted to different sterilization methods.

Table 4: Selected sterilization methods for testing the antimicrobial activity of the dressings.

	AbsorKi®	HemoKi	HidroKi®
PHMB	HHP	HHP	HHP
CHX	HHP	HHP	Steam and Pressure

Fig. 16 demonstrates the relative values for the optical densities (OD) of the incubated solutions containing bacteria obtained after 24 h of contact with samples loaded with PHMB and CHX, respectively. It is obvious that all dressings affected the bacterial growth by destroying the vast majority of the bacteria in the well-plate.

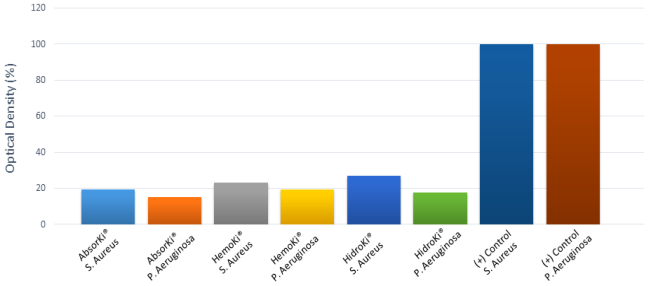

Figure 16: Relative values of the optical density of the incubation solutions containing S. aureus and P. aeruginosa, with PHMB and CHX, respectively The errors are the maximum ± standard deviations (n=3).

4. Discussion

After skin damage, the process of wound healing is very complex. It involves not only several biological processes and mechanisms but also the risk of contamination when the wound is in contact with pathogenic microorganisms [17]. Therefore, the production of medical products that protect the wound and accelerate the wound healing process is imperative. In this work, an effort was made to produce efficient drug loaded chitosan based wound dressings and study the effect of various sterilization methods on them. Several tests were conducted in order to assess the properties of the developed materials, namely AbsorKi®, HemoKi and HidroKi® before and after sterilization and, if possible, to choose the best sterilization method.

Due to its water uptake capacity, the swelling of WD has an important role in wound healing because it affects the amount of exudate that can be absorbed from the wound [18]. With the exception of HidroKi®, the other dressings with and without sterilization exhibit extremely high capacities for

water sorption, which is associated with their spongy texture which was achieved due to lyophilization [19]. All sterilization methods increased the swelling capacity of AbsorKi® and of HidroKi®, but decreased the swelling capacity of HemoKi. For HidroKi® it was observed that non-sterilized samples demonstrate very low swelling capacity, while both sterilization methods (steam and heat and HHP) improved significantly this property. the swelling of the samples AbsorKi® and HidroKi® sterilized by HHP increased compared to the non-sterilized samples. However, as observed for HemoKi, it is possible that the pressure and the increased temperature induced further cross-linking of the material. The matrix became denser and the porosity decreased resulting in lower water sorption than the unsterilized material. This fact is in agreement with the Flory theory and studies of other authors [20-23].

Wettability is a very important parameter for the function of WD because it influences the rate of fluid absorption by these products, especially for exudate wounds [24] and also affects the cells and microorganisms' adhesion [25]. In general, from the experimental data it was clear that the material is hydrophilic (low contact angle values) since chitosan is a biopolymer known for its hydrophilicity. Comparison of the contact angle values of sterilized and non-sterilized samples shows that that HHP decreased slightly the wettability of HidroKi® but overall, one may conclude that the sterilization process did not affect the wettability of the material. Moreover, the fact that the contact angle values maintained stable throughout the experiments indicates that the hydrogel is in equilibrium with the solution and there will be no further wetting of the surface.

WD should possess appropriate mechanical properties, such as adequate strength, stiffness and flexibility, because they must be stress resistant in order to withstand the normal stress encountered during their application and handling [26]. Overall the studied materials are weak and that can be fractured or be compressed easily. Such behaviour could be expected since there are other studies stating that it is common for porous structures to exhibit inferior mechanical properties compared to dense structures [26, 27]. The properties of AbsorKi® and HemoKi changed significantly after HHP, while for HidroKi® a small improvement was observed after steam and pressure sterilization. More specifically, the results obtained with AbsorKi® indicated gamma radiation, among all the other methods, worsened its properties. The values of YM indicate that the irradiation made the material less rigid (low TS value) and the BS was reduced to the half compared to non-sterilized samples. This fact might have happened because irradiation induced changes in the matrix of the hydrogel and caused massive chain scission. On the other hand, sterilization by HHP appears to improve the material's properties. The TS value is higher significantly, making the dressing capable of remaining intact for longer time while it is being elongated. These changes are a consequence of the high pressure that was applied to the sample (600 MPa) and can alter the intermolecular distances and modify hydrophobic interactions and electrostatic bonds [28], increasing the YM. After steam and pressure AbsorKi® became less rigid (lower YM) and the capacity to resist to load decreased (lower TS). Similar behaviour was observed for HemoKi. Once again, sterilization with gamma radiation has a negative effect on the material by lowering its resistance to strain. Sterilization by steam and pressure made HemoKi less brittle and stiff. Probably during the autoclaving process some thermal degradation occurred enhancing the mobility of the chains. Concerning the HHP effect, according to the value of TS and YM it is obvious that the material became more resistant and can withstand more stress until it raptures completely. Concerning HidroKi®, HHP almost had no effect on its mechanical properties. This characteristic could be explained by the fact that the samples are in contact with water during the sterilization; thus, the pressure is transmitted through the liquid and is applied uniformly in all directions. In contrast with the dry samples, the effects of steam and pressure on the hydrated samples are positive. Steam heat also did not lead to significant differences on YM and CS. Therefore, it is not possible to conclude about the best sterilization method for HidroKi®.

The SEM analysis revealed significant changes in the surface morphology of all samples after sterilization for all the different methods that were applied. HHP reduced the sponge-like character of HemoKi and AbsorKi®. Regarding HemoKi, the presence of genipin led to a more compact structure, which was affected by the conditions during the sterilization and most likely was further cross-linked. Important changes in the surface morphology of HidroKi® were observed for both steam and pressure sterilization and HHP. However, these sterilization methods benefited the swelling capacity of the material and did not cause any deterioration to its mechanical properties.

The results from the HET-CAM tests did not exhibit any signs of visual lysis, haemorrhage or coagulation which implies that none of the materials induces skin irritation.

Chronic wounds and large burns are susceptible to infection from pathogenic microorganisms. They can either be not very extensive and can be cured by local drug delivery or be more severe and their healing might require even surgical treatment [29]. Two widely used antiseptic drugs, PHMB and CHX, were chosen to be incorporated in our WD. In order to simulate the actual drug release from the wound dressings to the skin, all the drug release experiments were performed using a Franz cell diffusion system. Also, the time frame that was set for these experiments was the 48 hours since it is unlikely that wound dressings will be used for more than two

days on the wound. From the data gathered from all sterilized materials, it can be inferred that the drug release of both PHMB and CHX is controlled for the first 8 hours (except for CHX in HidroKi®). Significant differences were noted regarding the concentration of the released drug. This variation among the two drugs could be due to the affinity of each drug to the different materials. Two of the materials are lyophilized and the other is hydrated; thus, the drugs could favour more one of these environments. Lyophilization is commonly used for the introduction of pores on a surface. The freezing rate and the freezing temperature can affect the pore size and this can further affect the amount of drug that enters in the material [30]. For HemoKi, the presence of genipin might also affect the interaction of the drug molecules with the hydrogel. Furthermore, the differences between the sterilization methods, might be attributed to possible interactions or chemical bonds which were formed between the material and the drug molecules during the sterilization process. Since the materials are immersed in drug solution for the loading process, the drug-loaded dressing can be affected by the sterilization conditions which occur at the same time, such as increased temperature during steam and pressure sterilization and high pressure during HHP. The only method that does not require the drug loading process and the sterilization process to happen simultaneously is gamma radiation. The samples are irradiated (25kGy) after the lyophilization but the gamma rays can influence the chemical or physical bonds of the material and induce further cross-linking in the case of HemoKi. Finally, further modifications in the hydrogels' matrix may have occurred but they were not detected by the studied properties of the materials.

Microbiological tests were performed in order to evaluate and reassure the microbiological safety of the developed (WD). Infection spread has not been associated only with wound contamination from pathogens but also with medical devices. Terminal sterilization ensures a high safety of the products [31, 32]. Among the most commonly used sterilization methods are steam and pressure and sterilization by gamma radiation. HHP is a quite recent but very promising sterilization method. Therefore, sterility tests were conducted in order to assess the efficiency of this method. The obtained results indicated that HHP could successfully eliminate all microorganisms from the developed materials. The applied pressure is so high that can inactivate the cellular processes of pathogenic microorganisms.

Finally, the antimicrobial activity of the used drugs was evaluated by measuring the OD of an incubation solution consisting bacteria and the developed materials loaded with drug. Only one method per dressing was chosen for the test based on the drug release profiles and the properties of the raw material. The information gained from the OD showed that all the different drug loaded WD were able to inhibit the bacterial growth very effectively. For all the different conditions, AbsorKi® displayed very promising applications because it killed the vast majority of bacteria in the solution, although HemoKi and HidroKi® appeared to be very effective as well. This behaviour is possibly related with the nature of the material combined with the antimicrobial effect of chitosan. More specifically, dry dressings do not favour the adherence of bacteria on their surface while hydrated dressings are more susceptible to this process. Nonetheless, it is not easy to make safe conclusions about this claim without conducting further tests associating the produced dressings with their natural bioburden and the wound bioburden.

5. Conclusions

The objective of this work was to develop efficient and safe chitosan based drug loaded WD and evaluate the effect of the sterilization on the WD. Chitosan was the main component of all materials due to its biocompatibility and antimicrobial properties. The sterilization methods used were: steam and pressure, HHP and gamma radiation. The porous structure of dry dressings allows them to absorb a very high amount of fluid. Sterilization, especially HHP, improved the water uptake of AbsorKi® while the swelling of HemoKi decreased after sterilization suggesting that all methods induced further cross-linking of genipin. Concerning HidroKi®, the sterilization method that most improved the swelling was steam and pressure. Regarding the wettability of HidroKi® (the only that was possible to measure), it was found that none of the sterilization methods leads to significant changes. The mechanical tests revealed that all dressings have low resistance and elasticity. However, sterilization of AbsorKi® and HemoKi by HPP led to a remarkable improvement in the tensile strength and elasticity. For HidroKi® a small improvement in the properties was observed after steam and pressure sterilization. All sterilization methods caused changes in the surface morphology of the materials. The most impressive modifications were caused by HHP which led to compression of the dry dressings and thickness decrease. From the drug release of both drugs for the WD, one can conclude that, overall, the different sterilization methods increased the concentration of the released drug. The only case where both steam and pressure and HHP sterilization decreased this concentration was when HidroKi® was loaded with PHMB. None of the developed WD exhibits any sign of irritation and combined with drugs can be very effective against bacteria.

References

1. Dhivya S, Padma VV, Santhini E. Wound dressings - a review. Biomedicine (Taipei). 2015 Dec;5(4):22. doi: 10.7603/s40681-015-0022-9. Epub 2015 Nov 28. PMID: 26615539; PMCID: PMC4662938. Surname A and Surname B 2009 *Journal Name* **23** 544

2. Jones V, Grey JE, Harding KG. Wound dressings. *BMJ*. 2006 Apr 1;332(7544):777-80. doi: 10.1136/bmj.332.7544.777. PMID: 16575081; PMCID: PMC1420733.
3. Paul, Willi & Sharma, Chandra. (2004). Chitosan and Alginate Wound Dressings: A Short Review. Trends in Biomaterials and Artificial Organs. 18.
4. Koehler, Julia & Brandl, Ferdinand & Goepferich, Achim. (2018). Hydrogel Wound Dressings for Bioactive Treatment of Acute and Chronic Wounds. European Polymer Journal. 100. 10.1016/j.eurpolymj.2017.12.046
5. Mozalewska, Wiktoria & Czechowska-Biskup, Renata & Olejnik, Alicja & Wach, Radoslaw & Ulański, Piotr & Rosiak, Janusz. (2017). Chitosan-containing hydrogel wound dressings prepared by radiation technique. Radiation Physics and Chemistry. 137. 1-7. 10.1016/j.radphyschem.2017.01.003.
6. Liu, He & Wang, Chenyu & Li, Chen & Qin, Yanguo & Wang, Zhonghan & Yang, Fan & Li, Zuhao & Wang, Jincheng. (2018). A functional chitosan-based hydrogel as a wound dressing and drug delivery system in the treatment of wound healing. RSC Advances. 8. 7533-7549. 10.1039/c7ra13510f.
7. Hamedi, Hamid & Moradi, Sara & Hudson, Samuel & Tonelli, Alan. (2018). Chitosan Based Hydrogels and Their Applications for Drug Delivery in Wound Dressings: A Review. Carbohydrate Polymers. 199. 10.1016/j.carbpol.2018.06.114.
8. Karajanagi SS, Yoganathan R, Mammucari R, Park H, Cox J, Zeitels SM, Langer R, Foster NR. Application of a dense gas technique for sterilizing soft biomaterials. Biotechnol Bioeng 2011;108(7):1716–1725.
9. Galante, Raquel & Pinto, Terezinha & Colaço, Rogerio & Serro, Ana. (2017). Sterilization of hydrogels for biomedical applications: A review: STERILIZATION OF HYDROGELS. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 106. 10.1002/jbm.b.34048
10. Halpern JM, Gormley CA, Keech M, von Recum HA. Thermomechanical properties, antibiotic release, and bioactivity of a sterilized cyclodextrin drug delivery system. J Mater Chem B 2014; 2(18):2764–2772.
11. Abuhanoglu G, Ozer AY. Radiation sterilization of new drug delivery systems. Interven Med Appl Sci 2014;6(2):51–60.
12. Ratner BD, Hoffman AS, Schoen FJ, Lemons JE. Biomater Science: An Introduction to Materials in Medicine. Amsterdam, The Netherlands: Elsevier Science; 2012.
13. Sakar F, Ozer AY, Erdogan S, Ekizoglu M, Kart D, Ozalp M, et al. Nano drug delivery systems and gamma radiation sterilization. Pharm Dev Technol 2016:1–10.
14. Vetten MA, Yah CS, Singh T, Gulumian M. Challenges facing sterilization and depyrogenation of nanoparticles: Effects on structural stability and biomedical applications. Nanomedicine 2014;10(7):1391–1399.
15. Balasubramaniam, VM & Martinez-Monteagudo, Sergio & Gupta, Rockendra. (2015). Principles and Application of High Pressure--Based Technologies in the Food Industry. Annual Review of Food Science and Technology. 6. 10.1146/annurev-food-022814-015539.
16. Robles Linares Alvelais, José & Figueroa-Cavazos, J.Obedt & Chuck-Hernandez, Cristina & Siller, Hector & Rodriguez, Ciro & Martínez-López, José-Israel. (2018). Hydrostatic High-Pressure Post-Processing of Specimens Fabricated by DLP, SLA, and FDM: An Alternative for the Sterilization of Polymer-Based Biomedical Devices. Materials. 10.3390/ma11122540.
17. Giri, Jeevan. (2018). Factors Affecting Wound Healing. 10.13140/RG.2.2.32275.32804.
18. Rusanu, Alexandra & Tamaş, Alexandra & Vulpe, Raluca & Rusu, Alina & Butnaru, Maria & Verestiuc, Liliana. (2017). Biocompatible and Biodegradable Hydrogels Based on Chitosan and Gelatin with Potential Applications as Wound Dressings. Journal of Nanoscience and Nanotechnology. 17. 4584-4591. 10.1166/jnn.2017.14298.
19. Weska, Raquel & Jr, Wellington & Nogueira, Grínia & Beppu, Marisa. (2009). Effect of Freezing Methods on the Properties of Lyophilized Porous Silk Fibroin Membranes. Materials Research-ibero-american Journal of Materials - MATER RES-IBERO-AM J MATER. 12. 10.1590/S1516-14392009000200020.
20. Statistical Mechanics of CrossLinked Polymer Networks II. Swelling Paul J. Flory and John Rehner Citation: J. Chem. Phys. 11, 521 (1943); doi: 10.1063/1.1723792.
21. Pourjavadi A., Barzegar S., Zeidabadi F. Synthesis and properties of biodegradable hydrogels of κ-carrageenan grafted acrylic acid-co-2-acrylamido-2-methylpropanesulfonic acid as candidates for drug delivery systems. React. Funct. Polym. 2007;67:644–654. doi: 10.1016/j.reactfunctpolym.2007.04.007.
22. Peppas N.A., editor. Hydrogels in Medicine and Pharmacy. CRC Press; Boca Raton, FL, USA: 1986.
23. Wu J., Lin J., Li G., Wei C. Influence of the COOH and COONa groups and crosslink density of poly(acrylic acid)/montmorillonite superabsorbent composite on water absorbency. Polym. Int. 2001;50:1050–1053. doi: 10.1002/pi.728.
24. A review on wound dressings with an emphasis on electrospun nanofibrous polymeric bandages, Payam Zahedi , Iraj Rezaeian , Seyed-Omid, Ranaei-Siadat, Seyed-Hassan Jafari, Pitt Supaphol First published: 07 December 2009 https://doi.org/10.1002/pat.1625.
25. Simona Dimida, Amilcare Barca, Nadia Cancelli, Vincenzo De Benedictis, Maria Grazia Raucci, and Christian Demitri, "Effects of Genipin Concentration on Cross-Linked Chitosan Scaffolds for Bone Tissue Engineering: Structural Characterization and Evidence of Biocompatibility Features," International Journal of Polymer Science, vol. 2017, Article ID 8410750, 8 pages, 2017. https://doi.org/10.1155/2017/8410750.
26. Mazor, Eytan & Zilberman, Meital. (2016). Effect of gamma-irradiation sterilization on the physical and mechanical properties of a hybrid wound dressing: Effect of Gamma-Irradiation Sterilization. Polymers for Advanced Technologies. 28. 10.1002/pat.3854.
27. J. J. Elsner, A. Shefy-Peleg, M. Zilberman, J. Biomed. Mater. Res. B, Appl. Biomater. 2010, 93, 425–435.
28. Bolumar, Tomas & Middendorf, Dana & Toepfl, Stefan & Heinz, Volker. (2016). Structural Changes in Foods Caused by High-Pressure Processing. 10.1007/978-1-4939-3234-4_23.
29. Saghi Saghazadeh, Chiara Rinoldi, Maik Schot, Sara Saheb Kashaf, Fatemeh Sharifi, Elmira Jalilian, Kristo Nuutila, Giorgio Giatsidis, Pooria Mostafalu, Hossein Derakhshandeh, Kan Yue, Wojciech Swieszkowski, Adnan Memic, Ali Tamayol, Ali Khademhosseini, Drug delivery systems and materials for wound healing applications, Advanced Drug Delivery Reviews, Volume

127, 2018, Pages 138-166, ISSN 0169-409X, https://doi.org/10.1016/j.addr.2018.04.008.
30. Abitha M. Heimbuck, Tyler R. Priddy-Arrington, Benjamin J. Sawyer, Mary E. Caldorera-Moore , Effects of post processing methods on chitosan genipin hydrogel properties. Msc (2019), https://doi.org/10.1016/j.msec.2018.12.119.
31. Galante R, Rediguieri CF, Kikuchi IS, Vasquez PA, Colaço R, Serro AP, Pinto TJ. About the Sterilization of Chitosan Hydrogel Nanoparticles. PLoS One. 2016 Dec 21;11(12):e0168862. doi: 10.1371/journal.pone.0168862. PMID: 28002493; PMCID: PMC5176313.
32. Dick, H. & Schwenn, Oliver. (2000). Aseptic Production vs. Terminal Sterilization. 10.1007/978-3-642-59779-4_10.