PhD THESIS

Polymeric biomaterials: design, optimization, characterization and clinical application for wound management in small animal clinical practice

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Regenerative medicine promotes tissue restoration through materials that are easy to obtain and have excellent properties, but also which have the potential to accelerate healing. Many studies have shown that biopolymer-based materials can provide the conditions for proper healing. Both bacterial cellulose (BC) and alginate dressings appear to have properties that can be applied to wound healing. Both polymers have shown adequate results in wound healing processes and both have all the properties needed for a dressing. Additionally, they are extensively studied on laboratory animals and are currently used in human medicine, but there appears to be a scarcity of studies on wound management in small animal clinical practice. In this context, the first part of the thesis presents the biomedical potential of dressings based on bacterial cellulose (BC) and alginate as materials for wound healing.

Thus, the first part of the thesis describes the **current state of knowledge**, challenges and future use-cases of BC and alginate and their biomedical applicability in veterinary medicine. This part is divided into two main chapters that describe the main biomedical properties and applications of the two biopolymer-based materials.

The first chapter (**chapter 1**), **"Bacterial biofilms as wound healing dressing"**, published in Scientific Works. Series C. Veterinary Medicine (**BDI**), aimed to critically present the current knowledge on biopolymer-based materials as wound dressings for veterinary use. Several reports on properties and biocompatibility of biopolymer-based materials, in general, and alginate and BC, in particular, showed that they could be successfully used in veterinary medicine. However, alginate and bacterial cellulose are not yet introduced in current veterinary practice. Both polymers were studied extensively in many reports, in respect to their physical, chemical, and structural properties. The study concluded that both alginate and BC have transparent hydrogel structures, with good mechanical properties, porous network structure, and high absorption and release abilities. Also, they have a good biocompatibility and, although they have no antimicrobial activity, they have the ability to incorporate active components. Both polymers find increasing use-cases in wound healing management with satisfactory mechanical properties and good appearance. At the same time, healing and wound dressing properties were evaluated, but reports overlooked comparing them in regard to their use as wounds dressings in small animal clinical practice. Thus, the main bacteria species involved in biopolymer production, their growth conditions and discrimination among the properties of bacterial cellulose and alginate were presented.

Chapter 2 entitled "**Applications of bacterial-synthesized cellulose in veterinary medicine"** published in Acta Veterinaria Brno (**IF = 0.667, Q3**) reports the current state of knowledge, challenges and future practicability of BC and its biomedical applicability in veterinary medicine. It was focused on the main uses in regeneration and scaffold tissue replacement, and it showed the most promising results. There are several known practical applications for BC in the medical domain, but there was a lack of pertinent presentation of their application in veterinary practice. The main biomedical applications seemed to be as dental implants, wound healing dressings, dura mater, and tympanic substituents, bladder reconstruction, nerve regeneration, artificial cornea, bone, and cartilage regeneration. Although BC was extensively tested on laboratory animals and it is currently used in human medicine, its use is scarce in veterinary practice. Thus, the aim of this chapter was to summarize and critically present the main properties of BC, advantages, and disadvantages of its use in animal tissue engineering. It seems to show great promise in tissue bioengineering due to its diversity and versatility of biochemical and physical properties as a tissue engineering scaffold.

Thus, the **aim** of the PhD thesis was to obtain and apply biopolymer-based materials enriched with bioactive compounds to support and accelerate wound healing processes in small animal clinical practice

To achieve this goal, the following specific **objectives** were proposed:

(1) To employ Response Surface Methodology (RSM) to produce bacterial cellulose membranes with desirable morphological, mechanical, up-take, and release properties for biomedical use, in general, and as wound dressing material, in particular.

(2) To evaluate the effect of oven-drying against the pristine water-drained BC using the same methodology. The same properties of biomedical interest were compared.

(3) To perform a screening of active compounds from oregano, rosemary, parsley, and lovage ethanolic extracts obtained by microwave-assisted extraction and, the effect of various extraction parameters (ethanolic concentration, microwave power, extraction time, and repetition) upon their chemical profile, and bioactive potential.

(4) To assess the bioactivity of a material containing bacterial cellulose (BC) as a scaffold and ethanolic extracts of herbs form parsley, lovage, oregano, and rosemary. The obtained biopolymer is intended to be used as a bioactive material for biomedical uses as a wound dressing.

(5) To assess the treatment and outcome of wounds using a polymer-based material.

(6) Finally, to evaluate the healing process, over time by a non-invasive method. A smartphone application will be used for wound size measurement and documentation of wound closure.

In this, context the **original part** of the thesis is organized in three sections. The first section of the thesis assessed the "**Optimization of moist and oven-dried bacterial cellulose production for functional properties**", published in Polymers (**IF = 4.493, Q1**) and includes **chapter 3**. This study analyzed the optimal culture conditions of BC membranes and 2 types of processing: draining and oven-drying, and achieved **objectives (1)** and **(2)**. The aim was to obtain BC membranes with properties suitable for a wound dressing material. Several other studies have already employed Response Surface Methodology (RSM) to increase the yield of bacterial cellulose by determining optimum fermentation conditions and medium components. Thus, this study not only showed a validated production procedure for bacterial cellulose with biomedical applicability, but it also contains a complete comparison of two post fermentation processing methods.

However, to the best of our knowledge, no previous study has tried to develop a production procedure of bacterial cellulose pellicles with optimal properties for biomedical uses such as uptake and release capabilities. Additionally, only a few studies have analyzed the morphological and mechanical properties of oven-drying BC, and none its uptake and release ability. Oven-drying is a relevant processing method because it is cheap, and easy to perform, with no need for special equipment and/or reagents.

Two studies were carried out. In the preliminary study the medium (100 mL) was inoculated with varying volumes (1; 2; 3; 4; and 5 mL) and incubated statically for different periods (3; 6; 9; 12; and 18 days), using a full factorial experimental design. Thickness, uniformity, weight, and yield were evaluated. In the optimization study, a Box–Behnken design was used. Two independent variables were used: inoculum volume (X_1: 1; 3; and 5 mL) and fermentation period (X_2: 6; 12; and 18 d) to determine the target response variables: thickness, swelling ratio, drug release, fiber diameter, Tensile strength, and Young's Modulus for both dry and moist BC membranes. The mathematical modeling of the effect of the 2 independent variables was accomplished by response surface methodology (RSM).

Thus, the preliminary study showed that a combined 0.1 M NaOH and 3% NaOCl purification treatment was effective in whitening BC, and TEM imaging revealed that all bacterial cells were removed from BC pellicle matrix. Additionally, the inoculum volume and the harvest day were shown to significantly influence the thickness and uniformity of the membranes ($p < 0.008$ and $p < 0.0001$, correspondingly). An increase in the fermentation period significantly increased BC dry weight ($p < 0.002$), water content ($p < 0.0001$), and yield ($p = 0.002$), while the uniformity was not influenced by the inoculum nor the fermentation period.

We confirmed the general opinion that the fermentation period greatly influences the yield, uniformity, and mechanical properties of the bacterial cellulose membranes, but also found that: the volume of inoculum significantly influenced the swelling behavior of bacterial cellulose; moist bacterial cellulose samples swelled significantly faster than oven-dried pellicles, suggesting that the oven-drying affects the swelling ratio of bacterial cellulose by probably modifying the morphology of the pellicle network. A gradual drug release was observed throughout 72 hours, with a significant difference between the two types of membranes throughout the tested period. Moist samples had up to three times higher drug release capacity, than dried pellicles, suggesting that the oven-drying processing negatively affects the drug release ability of bacterial cellulose.

We managed to validate the production method for all the 5 response characteristics of moist BC. For a "wound dressing"-use scenario of the two types, we would recommend the moist type BC because of its fibrillar network that was able to incorporate bioactive compounds (half-swelling time of 1.930 h) and optimally release them (drug half-release time of 3.678 h). This would lead to a hastening healing of wounds. Additionally, the obtained moist BC has good tension strength (4.770 MPa) and low elongation at break (13.305 MPa), which would make the BC an easy to work with material. Additionally, BC maintains proper moisture by either absorbing exudates or releasing and actively participate in tissue regeneration.

Additionally, both tested variables (harvest day and inoculum) seemed to negatively influence the diameter of the fibrils, with the inoculum volume having a greater influence than the harvest day. The obtained models were validated with new experimental values, and confirmed for all tested properties, except Young Modulus of oven dried BC. It seems that by setting the optimization goals in line with the requirements of a "wound dressing"-use scenario for bacterial cellulose, we obtained and validated the following values for the fermentation parameters: an inoculum volume of 5 and a harvest day of 16 for moist bacterial cellulose, and an inoculum volume of 4 mL and a harvest day of 14 for oven-dried pellicles.

Chapter 4 includes a screening of "**antimicrobial properties of bacterial cellulose films enriched with bioactive herbal extracts obtained by microwave-assisted extraction"** published in Polymers (**IF = 4.493, Q1**)**.** The study aimed at assessing the bioactivity of a material containing bacterial cellulose (BC) as scaffold and ethanolic extracts of herbs form *Lamiaceae* and *Apiaceae* families, and accomplished **objectives (3)** and **(4)**. The obtained polymer was intended to be used as a bioactive material for biomedical uses as wound dressing. Natural extracts were proposed as active substances, which were extracted by using a "green" environmentally friendly extraction procedure. Thus, microwave-assisted extraction was used to extract bioactive compounds from rosemary, oregano, lovage, and parsley. The total phenolic content (TPC), antioxidant (AA), and antimicrobial activity against *S. aureus*, *E. coli,* and *C. albicans* were evaluated. Additionally, the effect of various extraction parameters (ethanolic concentration, microwave power, extraction time and repetition) were assessed upon the chemical profile, and potential bioactivity of the extracts.

Three microwave-assisted extraction protocols were used to obtain ethanolic extracts from 4 culinary herbs (oregano, rosemary, lovage, and parsley) as natural bioactive substances to be loaded into the BC. The protocols varied in terms of solvent concentration, microwave power, extraction duration and repetition. Both TPC and AA of the selected herbs varied significantly, with rosemary having overall the highest TPC and AA, followed by oregano, lovage, and parsley. In this sense, a significant linear relationship between TPC and AA was obtained. The 4 herbs all showed significant antimicrobial activity, which varied by herb, extraction procedure, and tested microorganism. Rosemary extracted with 60% ethanol had the highest antibacterial activity against both *S. aureus* and *E. coli*, while the extract obtained with 80% ethanol presented the highest activity against *C. albicans.* Lovage and parsley had a lower antimicrobial activity. All extraction parameters seem to significantly influence the bioactivity of the extracts, with the solvent concentration having the most evident effect.

PCA was used to decide which extracts to be loaded onto the BC because the results obtained for TPC and bioactivity were not that straightforward as to fundament this decision. One common extraction protocol was chosen for the 4 herbs (80% ethanol, 800 W, 10 s, and 5 repetitions) that could produce extracts with the highest overall antimicrobial activity against the 3 tested microorganisms.

Then, BC with desired mechanical properties and an optimal 3D network structure that allowed the loading of herbal extracts was obtained. The BC obtained in this study had a good transparency after the purification treatment and an optimal 3D network that allowing the loading of herbal extracts. The median cellulose fiber diameter was of 48.14±19.92 nm, while the drained pellicle withstand a maximum load of 2.77 ± 0.74 N and it presented a tensile strength of 2.31 ± 0.61 MPa. Afterwards, the bioactive potential of BC enriched with the four ethanolic extracts was assessed. FT-IR was used to demonstrate that the presence of the extracts in BC structure did not induce many distinct spectral characteristics that could be directly assigned to the molecular structure of the extract components. Results showed that BC enriched with rosemary extract exhibited the highest antibacterial activity against *S. aureus*. However, *E. coli* and *C. albicans* seemed to be resistant to the enriched BC samples.

To the best of our knowledge this study tackles little studied issues in the field of natural polymers, especially BC loaded with herbal extracts. Thus, we have assessed the effects of microwave-assisted extraction upon the bioactivity of rosemary, oregano, lovage, and parsley ethanolic extracts, aimed at finding the most suitable procedure for an active substance. Although many studies have evaluated the effect of these parameters on the phenolic content and antioxidant activity of ethanolic extracts, to the best of our knowledge, there are no previous studies that assessed the influence of microwave assisted extraction parameters (ethanol concentration, microwave power, extraction repetition and extraction period) on the antimicrobial activity of herbal extracts against tested microbial strains.

Additionally, we showed that the loading of herbal ethanolic extracts into BC seems to be a promising technique in obtaining a biodegradable membrane with good antimicrobial properties. However, these are only proof-of-concept results that set the stage for further studies. Such as the extrapolation of *in-vitro* results needs to be validated *in-vivo* for biomedical uses.

The use of bacterial cellulose (BC) as scaffold for active membranes is one of the most interesting applications especially for biomedical industry. However, currently, there are few studies evaluating the potential of incorporating herbal extracts into various biomaterials, including BC.

The last chapter (**chapter 5**) describes the "**Clinical benefits of using a smartphone application to assess the wound healing process in a feline patient**" published in Topics in Companion Animal Medicine (**IF = 1.631, Q3**). This part of the PhD thesis desired to achieve **objectives (5)** and **(6).** The aim of this chapter was to determine whether digital imagery can be employed in veterinary medicine to measure and analyze wound healing dynamics. A smartphone application (ImitoMeasure) was used to capture, measure, and analyze the metatarsal wound images in a cat patient. Additionally, this study describes the treatment of a cat with a severe chronic metatarsal wound and extensive soft tissue loss using a commercially available silver calcium alginate dressing (Askina Calgitrol® Ag, B. Braun). The dressing was changed every 2 days, in the first week of treatment, and then every 3 days until the cat was discharged, 21 days later. Granulation tissue formed rapidly, from the 4th day of treatment and continued to expand in the entire wound bed; epithelization process started since the 16th day of treatment and mature scar tissue could be observed 21 days post-injury. During the treatment, alginate was easily removed, without injuring the new granulation tissue. New granulation tissue continued to develop, no exudate or any sign of infection were noticed, and a significant reduction of the wound size was observed during treatment. Complete wound healing was observed within 3 weeks, when the cat was discharged. Usually, the wound healing rate for this type of injury, with large tissue loss, implies extensive treatment and needs more time for complete closure. These results make alginate a promising choice as dressing in wounds with large tissue loss and could be used in daily veterinary clinical practice.

This study indicates an alternative use of alginate as a dressing to promote wound healing. Alginate dressing has the ability to absorb exudates, stimulate tissue regeneration, with proved antibacterial properties due to silver, and is able to generate proper healing. Thus, results reveal the benefits of alginate in wound healing.

The smartphone app was used to evaluate the wound area, width, length, and circumference at the time of topical treatments. Further analysis of the measurements taken by the ImitoMeasure app revealed significant correlations among all analyzed parameters. The day of treatment was inversely correlated with all the parameters of the wound, showing the healing progress over time. Also, the width was the most influential parameter ($p \leq 0.05$) when assessing wound area. Thus, the app provided a non-contact, easy to use, and accurate smart wound measurement solution.

Therefore, a proper evaluation technique for routine veterinary practice needs to be time- and cost-efficient, accurate and sensitive, quick, to minimize animal discomfort and avoid sedation, and useable by any clinician. Even though, in our case, traditional measurements were not used, due to the invasiveness of the method; also, wound contamination was minimal because the QR code was not placed in direct contact with the wound surface, as in traditional measurement.

Thus, smartphone apps can provide veterinary clinicians a non-contact, easy to use, reliable and accurate tools for wound management. In veterinary medicine sedative restraint is usually necessary for traditional manual measurements. Thus, a noninvasive measurement technique is preferred. Moreover, ImitoMeasure application is free and is able to photo-document and record the wound healing dynamics over time. However, wound measurement apps have some limitations. The reliability of the image is limited to its quality (lighting conditions and lack of movement) and to the operator variability when manually tracing the wound perimeter. Additionally, incorrect wound measurements may occur due to wound size, curved body surfaces or wound not fully visible to the camera.

Therefore, based on our results, we could **conclude** that BC is suitable for a high valuable wound dressing due to its remarkable structural, physical, and chemical properties. At the same time, the addition of herbal ethanolic extract to BC seems to be a promising technique in obtaining a biodegradable membrane with proven antimicrobial properties granted by natural antimicrobials. However, these are only proof-of-concept preliminary results that set the stage for further studies that need to properly describe the drug-release dynamic of the bioactive BC. Additionally, the extrapolation of *in-vitro* results needs to be validated *in-vivo* for biomedical uses.

At the same time, results showed that silver alginate dressings offered proper support for wound healing in our feline patient. It provided a proper environment by absorbing excess exudate and promoted epidermal regeneration and healing. Also, it was easily and pain-free removed without destroying the newly formed granular tissue. The silver in its structure has antibacterial properties which inhibits wound infections and is non-toxic to tissues.

The smartphone app. was proved to be time saving, easy to use, and enabled the recording and evaluation wound progression. Another advantage is the non-invasive nature of this application, fact that improves quality and reduces human errors in clinical wound-care documentation. The use of mobile applications simplifies the assessment of wound measurement parameters, eliminates the use of additional measuring devices, and accelerates data processing.

Thus, based on these results, we can **recommend** that further research on the use of BC and BC composites in wound healing is required to extend its healing ability on several types of skin lesions. These results are promising, highlighting the clinical potential of BC in the future, in skin tissue repair. Also, most studies were performed only at experimental level and further research is needed for BC to enter clinical veterinary practice. Thus, further studies are needed because there is a lack of information on use of bacterial cellulose in current veterinary practice and studies showed good results, although some were performed only at experimental level, its biomedical potential in veterinary medicine should be demonstrated.

Additionally, alginate dressings offered proper wound healing in our feline patient by providing a proper environment, but further studies are needed to validate its applicability and extend the possibility to enter in the current veterinary practice. At the same time, phone applications for wound assessment could be used in everyday clinical practice to evaluate skin injuries advancement, being cheap and easy to use. Thus, further studies in these directions are needed to confirm the effectiveness of wound evaluation and accurate assessment. Further research and practical case studies are needed to fully disclose the clinical applications of smartphone applications.