Uptake of PHMB in a bacterial nanocellulose-based wound dressing: A feasible clinical procedure

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ABSTRACT

Background: With the increase of antimicrobial resistance in recent decades, other methods of preventing and fighting infections must be considered. Burn patients, whose wound areas are often extensive, are especially prone to wound infections. The loading of bacterial nanocellulose (BNC) with antiseptics has already been successfully performed but unfortunately, the described procedure is time-consuming and thus not applicable in a clinical emergency setting. Therefore, a clinically feasible approach was established.

Material and methods: Sheets of BNC-based wound dressings were placed into antiseptic solutions containing PHMB (Prontosan® and LAVANID® 2) and were left to soak for up to two hours. At different time points, samples were analysed for their concentration of PHMB and antiseptic efficacy.

Results: Within 30min, clinically relevant concentrations of PHMB were achieved in the BNC-based wound dressing. The 30-min PHMB uptake for Prontosan® and LAVANID® 2 resulted in concentrations of 0.05% and 0.019%, respectively. Samples from the PHMB loaded dressing showed a dose dependent antiseptic efficacy for Staphylococcus aureus.

Conclusion: This experiment showed that the loading of BNC-based wound dressings with PHMB-containing antiseptics was achieved by a simple and quick procedure. According to studies a PHMB concentration of 0.001% can already inhibits all bacterial growth, indicating that the concentrations of PHMB in the BNC-based wound dressings after 30min are higher than the minimal inhibitory concentration and the antiseptic efficacy after 120min loading analysed by an standardized bacterial disk diffusion assay was shown to be comparable to the clinically used Suprasorb® X+PHMB wound dressing.

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1. Introduction

In 2014, the World Health Organization (WHO) recognized the problem of increasing multidrug-resistant microbes and declared it one of the greatest threats to global health [1]. In the US 23,000 people die as a result of infections by multidrug-resistant bacteria annually [2]. Patients with a suppressed immune system, elderly patients, or patients with large wound areas such as burn victims are at particular risk of infections with multidrug-resistant bacteria. Burn victims have an especially high mortality when associated with infections and the risk of infection is elevated with a prolonged hospital stay [3]. With increasing antimicrobial resistance, there is an urgent need for methods other than antibiotics to combat infections. In 2012, Selig et al. published an online survey about the ideal appearance of a wound dressing for burn patients that should be non-adhesive, absorbent, and should have antimicrobial properties [4].

Wound dressings with antimicrobial properties have been used clinically for some time, however it must be stated that burn wounds are frequently colonized by bacteria that produce biofilms impenetrable to antibiotics, thus rendering an antibiotic ineffective [5]. Silver-coated bandages, for example, are standard equipment in hospitals, but they still carry some risks like symptoms of silver toxicity such as elevated liver enzymes, leukopenia, or argyria-like symptoms [6–8]. Therefore, novel approaches are needed to address the complications associated with traditional wound dressings.

Bacterial nanocellulose (BNC) is a biomaterial produced by Komagataeibacter xylinus, which creates a fleece of fibrils of nanocellulose. In comparison to synthetic fibres, BNC possesses characteristics of a native nanofibrillar structures, a higher content purity, and higher tensile strength. Additionally, because of its hydrophilic properties, BNC has gained attention as a remarkable hydrogel for wound dressing production [9]. Due to these characteristics, BNC is not only fully biocompatible but can also protect a wound from excessive fluid loss, hence facilitating and accelerating the wound healing process and reducing pain associated with dressing changes [10,11].

This hydrophilic property enables the uptake of aqueous solutions such as antiseptics like polyhexamethylene biguanide, otherwise known as polyhexanide or PHMB. Its structure is similar to antimicrobial peptides, that are produced by cells within a wound to fight infection and it has been used over 60 years in a wide array of antiseptic applications [12].

Studies by Wiegand et al. and Moritz et al. have shown that BNC has excellent qualities not only in the uptake of antiseptics such as octenidine, povidone-iodine (PI), or PHMB but also in the release of these substances. Loading of BNC was performed by immersing samples of BNC in solutions of PI or PHMB and shaking them in an orbital shaker at 70 rpm for 48 h at 20 °C [13,14].

These studies have demonstrated how well BNC can be loaded with antiseptic substances and deliver them but the procedure is currently not applicable in everyday clinical use. When a patient with severe burns is admitted to the hospital, time plays a major role, and an adequate wound dressing is needed immediately. Forty-eight hours are too long for a clinician to prepare a wound dressing, and the use of an orbital shaker might be impractical especially if a significant amount of wound dressing is needed.

To address this problem, we tested a simpler method of loading BNC with PHMB that is efficient and easy to perform in an emergency room setting. Additionally we tested the antiseptic efficacy of the loaded BNC-based wound dressing on an adapted standardized bacterial disk diffusion assay for Staphylococcus aureus.

2. Material and methods

The BNC used was 10 × 10 cm sheets of epicitehydro by QR Skin (Ref-No. 800003-M02B) and Suprasorb® X+PHMB from Lohmann & Rauscher GmbH & Co. KG. PHMB concentration of 20% was purchased from FAGRON GmbH & Co. KG, LAVANID® 2 (0.04% PHMB) from SERAG-WIESSNER GmbH & Co. KG, and Prontosan® (0.1% PHMB; 0.1% Undecylenamidopropyl betain) from B. Braun Melsungen AG.

2.1. Spectrophotometry of PHMB

Ultraviolet/Visible (UV/Vis) spectrophotometry was used to quantify the antiseptics, as the best wavelength for analysing the PHMB molecule is between 234 and 236 nm [15,16]. The quantification experiment was performed with different concentrations of PHMB solution using a special UV-readable 96 well plate (CLS3635 SIGMA Corning® UV-Transparent Microplates). To perform the readings the Infinite® 200 PRO (TECAN Trading AG, Switzerland), a spectrometry plate reading device, was used. A wavelength of 235 nm was used and the calibration curves were recorded in the range of 20–100 μg/ml PHMB equating to 0.01–0.05% (Fig. 1).

2.2. Loading experiment

Two 10 × 10 cm sheets of epicitehydro were each placed into individual conventional steel kidney dishes and covered with either 200 ml of Prontosan® or LAVANID® 2. The immersed

![Fig. 1 – Quantification experiment of PHMB; Calibration curve with linear equation (γ=14.733x –0.0094) and correlation coefficient (R²). (PHMB — Polyhexanide).](image-url)
sheets were incubated at room temperature without shaking. After 10, 30, 60, 90 and 120 min two 8 mm diameter punch samples were taken from each sheet of epicate\textsubscript{hydro} (Fig. 2). For the epicate\textsubscript{hydro} sample immersed in Prontosan\textsuperscript{©}, the liquid was eluted by centrifugation through a membrane spin column (200 \textmu m pore size, 5 min at 1000 rpm); for the epicate\textsubscript{hydro} sample immersed in LAVANID\textsuperscript{©} 2, the liquid was eluted by pressure. These two different methods were used since pre-testing had revealed that the filter of the centrifuge retained a great part of PHMB from LAVANID\textsuperscript{©} 2. The same amount of liquid (75 \textmu l) was eluted using the different methods. The equation obtained via the calibration curve was used to calculate the concentrations of PHMB in the solutions extracted from the epicate\textsubscript{hydro} after incubation with either LAVANID\textsuperscript{©} 2 or Prontosan\textsuperscript{©}.

2.3. Method for standardized bacterial disk diffusion assay

Antiseptic efficacy of PHMB loaded epicate\textsubscript{hydro} material was measured by an adapted standardized bacterial disk diffusion assay for S. aureus. Briefly, the procedure was performed based on the protocol from Clinical & Laboratory Standards Institute [17] and Deutsches Institut für Normung (58940-3) [18]. S. aureus (ATCC 6538) cells were diluted to match the 10\textsuperscript{6}cfu/mL and cultured on medium. Sheets of epicate\textsubscript{hydro} were loaded with the selected antiseptics for 10, 30 and 120 min as described above (Fig. 6) and 8 mm punch samples were extracted, in duplicate. The epicate\textsubscript{hydro} loaded punches were placed on the surface of the agar plates and incubated overnight at 37°C. Images were acquired from the cultured plates and the area from each zone of inhibition (ZOI) was measured in pixel units using the software ImageJ.

3. Results

The results indicate that Prontosan\textsuperscript{©} (0.1\% PHMB) easily penetrates sheets of epicate\textsubscript{hydro} even when incubated without shaking. After 2 h, the concentration of PHMB in epicate\textsubscript{hydro} sheets reached 0.076\% (95\% of possible maximum, Fig. 3A and B). After 30 min, the concentration of PHMB was higher than 0.05\%. The water content initially present in epicate\textsubscript{hydro} is at least 95\% and it swells due to further uptake of fluid. The maximum possible uptake of a PHMB solution into epicate\textsubscript{hydro} yielded 80\% of the original concentration (data unpublished). Therefore, the maximal PHMB uptake from Prontosan\textsuperscript{©}, which has a PHMB concentration of 0.1\%, yielded a final concentration of 0.08\%.

LAVANID\textsuperscript{©} 2 in epicate\textsubscript{hydro} reached a PHMB concentration of 0.019\% after 30 min and after two hours the concentration increased to 0.024\% (Fig. 4A). Comparing these results to the possible maximum uptake values (100\% would be 0.032\%), they corresponded to an uptake of 59\% after 30 min and 76\% after 2 h (Fig. 4B). Comparing the uptake of PHMB from the two antiseptic solutions into sheets of epicate\textsubscript{hydro} showed an excellent relative uptake for both Prontosan\textsuperscript{©} and LAVANID\textsuperscript{©} 2 with a slightly better uptake from Prontosan\textsuperscript{©} (Fig. 5).

Antiseptic efficacy of epicate\textsubscript{hydro} material from different time points of loading with PHMB was measured by an adapted standardized bacterial disk diffusion assay for S. aureus and compared to the commercially available Suprasorb\textsuperscript{©} X+PHMB (see Fig. 6) wound dressing. The area of the ZOI increases with the time of loading and shows a comparable size for LAVANID\textsuperscript{©} 2 and Prontosan\textsuperscript{©} after 120 min of loading. The antiseptics loaded on epicate\textsubscript{hydro} hold different concentrations of PHMB (Prontosan\textsuperscript{©}’s PHMB content (0.1\%) is 2.5 times higher than LAVANID\textsuperscript{©} 2). Accordingly, the results for the area of the ZOI, for incubation periods 10- and 30-min, displays an equivalent ratio (Fig. 6B). This ratio was not found when for the 120 min materials showing comparable results for both treatments. After this time of loading, both materials show an antiseptic activity comparable to Suprasorb\textsuperscript{©} X+PHMB.

4. Discussion

Our data clearly showed that epicate\textsubscript{hydro} sheets can be loaded with sufficient amounts of PHMB from antiseptic solutions within a time period that is feasible in emergency situations. When considering such a setting, 30 min is a practically reasonable time to prepare a wound dressing, considering the
interval from preclinical notification or arrival of an ambulance to when other procedures are done, and wounds are treated. The experiments of Hirsch et al. showed that at a 1% dilution of Prontosan, equivalent to a PHMB concentration of 0.001%, the growth of *S. aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* could not be detected [18]. When loading epicitehydro with Prontosan or LAVANID 2 for 30min, PHMB concentrations reached 0.05% and 0.019%, respectively and are far higher than the minimal inhibitory concentration as reported by Hirsch et al. [19]. Additionally, when considering a surgical setting, 30min are sufficient to develop a PHMB infused wound dressing efficiently. If the BNC-based wound dressing is immersed into the antiseptic solution when the patient reaches the operating theatre, more than 30min will pass until wound debridement is completed and the wound is ready for dressing, making it possible to achieve a sufficient PHMB concentration of the wound dressing. In summary, a 30-min period was found to provide ample time to load this specific BNC-based wound dressing in either emergency or surgical settings.

For further and planned wound dressing changes, time may no longer be an issue, and even higher concentrations of PHMB can be achieved in epicitehydro. Additionally, the simple preparation of merely immersing the BNC sheets in an antiseptic solution of the clinician’s choice does not require specialized equipment or training.

By loading epicitehydro with an antiseptic solution containing PHMB, an ideal wound dressing for burn injuries can be created. Considering the demands of international burn specialists, published by Selig et al. [4], all requirements are met with this method. This loaded BNC-based wound dressing is not only absorbent and non-adhesive but also possesses the antimicrobial properties demanded by burn specialists, thus making it ideal for the topical treatment of burn wounds.
In addition, during the loading experiment we observed that BNC samples remained inherently stable with a smooth surface even after the incubation period and procedure was finished. This observation is in accordance with data of Wiegand et al. where after loading their BNC fleece with PHMB it remained form stable with a smooth surface and no change in colour. The same group found that the BNC fleece loaded with PHMB exhibited compressive strength values comparable to those of native BNC fleece [13] which indicates that the BNC sheets do not interact with the antiseptic solution in a manner that alters their physical appearance or compromises their mechanical properties.

The drug release profile and kinetics of PHMB were not investigated in this experiment as they have been described in detail by Moritz et al. and Wiegand et al. For their release experiments, BNC samples loaded with PHMB were transferred from the loading solution into 20ml of PHMB buffer. The samples were incubated at 32°C at 70rpm. Up to 48-h aliquots of the supernatants were collected at various time points and analysed. The concentration of the released PHMB was...
quantified by UV/Vis spectrophotometry. The medium remained colourless; therefore, the release of PHMB was not visibly observed. Within the initial 8h, a fast release was observed nearing 67%. Furthermore, equilibrium conditions were reached after 24h, while after 48h 87% of PHMB was released [13,14].

Since the antiseptic substance PHMB exhibits excellent biocompatibility and low cytotoxicity [12,20,21], long-term contact of the PHMB infused BNC wound dressing with a wound is preferred over a short-time rinsing. By using PHMB loaded wound dressings, prophylactic antibiotic use and antibiotic treatment of wound infections may be reduced, thereby preventing the further development of antimicrobial resistance.

Regarding the adapted standardized bacterial disk diffusion assay for S. aureus, Koburger et al. [22] showed a high antibacterial efficacy of PHMB against S. aureus, with a minimum bactericidal concentration (MBC) of 1mg/L after 24h. Our results directly correlate with the difference on the PHMB content on the chosen antiseptics. Both LAVANID®2 and Prontosan® punches from epicitehydro for 10min and 30min incubation period, showed proportionate area of inhibition compared with its original PHMB concentration. Prontosan®’s result mostly relies on the higher concentration of PHMB and the excipients in the material. Hirsch et al. [23] showed that Prontosan® could maintain its antimicrobial activity in combination with a higher number of wound dressings compared to Lavasept®, a PHMB-based product similar to LAVANID® 2. However, the same ratio could not be detected for the 120min incubated epicitehydro, which inhibited a comparable area despite of different PHMB concentrations for LAVANID® 2 and Prontosan®. This could be due to an overestimation of the highly variable LAVANID® 2 area results.

Although the concentration of PHMB in Suprasorb® X +PHMB is 7.5 times higher when compared with LAVANID® 2 and 3 times higher compared with Prontosan®, this difference is not translated on the size of the ZOI after 120min achieving only around 30% smaller areas. The inhibition area induced by Suprasorb® X+PHMB compared to the one by epicitehydro incubated with LAVANID® 2 and Prontosan® for 120min were unexpectedly similar. Recently published data for the antibacterial activity of Suprasorb® X+PHMB in comparison to Prontosan® in a treatment against S. aureus showed similar results [24]. Thus, the results presented in our study indicate that epicitehydro takes up and releases the tested PHMB based antiseptics in a way that efficiently promotes their activity against S. aureus in the type of assay used.

5. Conclusion

Our experiments demonstrate that the loading of epicitehydro with PHMB-containing antiseptics can be performed very fast, without any specialized equipment or training resulting in a wound dressing with an in vitro antiseptic efficacy against S. aureus comparable to the clinically used Suprasorb® X +PHMB dressing. Thus, this method is suitable for use in clinical emergency settings, which demand prompt treatment with infection-reducing methods and biocompatible materials. To prove our claims we will perform a clinical trial.

Limitations

Limitations of the study are that the antiseptic efficacy was only tested in vitro but was comparable to Suprasorb® X +PHMB. This cellulose material shows a good antiseptic activity in clinical use [25,26] but has a different matrix structure and lower water content. Therefore, future clinical studies with PHMB soaked epicitehydro in burn or chronic wounds should allow a better comparison. The combination of epicitehydro and PHMB solutions results in comparable costs compared to Suprasorb® X+PHMB but allows for a higher flexibility in dosing of the PHMB and size of the epicitehydro wound dressing that is available in sizes of 10×10cm, 10×15cm, 20×20cm and as a face mask. Creating a loaded BNC-based wound dressing is not time consuming or labour intensive but the storage temperature of the wound dressing should not exceed room temperature with an optimal temperature between 5-30°C and therefore has to be monitored.

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Conflict of interest

Martin Funk is part of the company QRSkin GmbH whose product epicitehydro was used for the experiment.

References


