Comparing two polymeric biguanides: chemical distinction, antiseptic efficacy and cytotoxicity of polyaminopropyl biguanide and polyhexamethylene biguanide

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In this study, polyaminopropyl biguanide (PAPB) was compared to the molecularly closely related polyhexamethylene biguanide (PHMB) with respect to chemical relationship, antiseptic efficacy and cytotoxicity in vitro. Cytotoxicity for human keratinocytes (HaCaTs) and murine fibroblasts (L929) was determined according to ISO EN 10993-5 for both substances. Antimicrobial efficacy tests were performed via determination of the MBC, quantitative suspension method for substances and investigation of two PAPB- or PHMB-containing dressings against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa, according to international standards. Prior mass spectrometry was performed for chemical differentiation of the investigated substances. PHMB showed high toxicity even in low concentrations for both tested cell lines and a high antimicrobial efficacy against S. aureus and E. coli. In the case of PAPB, no or only low cytotoxicity was detected after 72 h, whilst comparable antibacterial features are lacking, as PAPB showed no relevant antimicrobial effects. Even though chemically closely related, PAPB proved to be ineffective in bacterial eradication, whilst PHMB showed a high efficacy. The discovery and establishment of safe and effective alternative antiseptics are important issues for the treatment of infected wounds. In particular, rising bacterial resistances to established agents, as well as ongoing discussions of potential toxic or carcinogenic effects emphasize this necessity. Nevertheless, the presented results highlight that even small changes in the chemical structure of related agents such as PHMB and PAPB can dramatically affect their efficacy and, therefore, need to be carefully distinguished and assessed side by side.

INTRODUCTION

In Western countries, lifestyle diseases such as diabetes, arterial hypertension and vascular diseases are widespread, resulting in major challenges for patients as well as caregivers, and increasing costs for healthcare systems. Associated with these disorders is a rising incidence of impaired wound healing, which is characterized by a chronically inflamed wound bed and bacterial infection (Gottrup et al., 2013). Common bacterial strains found in chronic wounds are Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli, often residing in biofilms which are increasingly resistant to antimicrobial therapy (Lipsky & Hoey, 2009). Thus, antiseptic wound dressings and agents are becoming increasingly important in the management of chronic and infected wounds (Heyer et al., 2013; Lipsky & Hoey, 2009).

Antiseptics and/or antimicrobials incorporated into dressings, such as alginates and foams, enable topical treatment directly at the site of infection. These present an opportunity to reduce the total amount of antiseptic substance needed for eradication and minimize the potential for systemic toxicity. The resulting avoidance of using systemic and/or local antibiotics possibly reduces the development of antibiotic resistance (Lipsky & Hoey, 2009). Besides the requested broad microbicidal spectrum with rapid onset and long-lasting effect, antiseptics should not be toxic to host tissue. To establish a parameter measuring and...
combining these two features of wound antiseptics, Muller & Kramer (2008) introduced the biocompatibility index (BI) defined as the ratio of the concentration at which 50% of murine fibroblasts are damaged and the microbialic effect produces at least 3 log_{10} reduction (99.9%). Amongst common antiseptics, oxicidine dihydrochloride and polyhexamethylene biguanide (PHMB) were stated to be the most suitable agents with a BI >1, representing antiseptic substances with effective microbialic activity and comparatively low cytotoxicity (Muller & Kramer, 2008).

Today, PHMB is widely used as an antimicrobial agent not only in medicine, but in cosmetics and the environment as well, e.g. as a water disinfectant. Since being a strong base and positively charged cationic biocide, the antiseptic potential of PHMB relies on interaction with acidic, negatively charged phospholipids in the bacterial membrane as well as interaction with bacterial chromosomes after penetration of the membrane barrier. This results in the elimination of micro-organisms through increased fluidity, permeability and loss of integrity as well as chromosome condensation and the disruption of bacterial metabolism when transferred into the bacterial cell (Chindera et al., 2016; Hubner & Kramer, 2010). PHMB is effective against planktonic Gram-positive and Gram-negative bacteria as well as biofilms and a variety of other species such as Saccharomyces cerevisiae, Candida albicans and Acanthamoeba (reviewed by Kaehn, 2010). Concentrations of 0.01 %, 0.02 % and 0.04 % PHMB are commonly used in wound antiseptics. Additionally, wound dressings containing PHMB have been shown to reduce bacterial burden and chronic wound pain in vivo (Eberlein et al., 2012).

In contrast to silver (Sütterlin et al., 2012), no bacterial resistance against PHMB has been described yet (Wessels & Ingmer, 2013). Additionally, it is stated to be ‘practically non-toxic’, well tolerated on skin, eyes, nose, ciliated epithelium and wounds, and showed no sensitizing or photosensitizing effects in animal tests (reviewed by Hubner & Kramer 2010). However, PHMB was recently under discussion for showing a certain carcinogenic effect and was proposed to be classified as carcinogenic Carc 2 (Henderson et al., 2014). However, PHMB was recently under discussion for showing a certain carcinogenic effect and was proposed to be classified as carcinogenic Carc 2 (Henderson et al., 2014). PHMB is effective against planktonic Gram-positive and Gram-negative bacteria as well as biofilms and a variety of other species such as Saccharomyces cerevisiae, Candida albicans and Acanthamoeba (reviewed by Kaehn, 2010). Concentrations of 0.01 %, 0.02 % and 0.04 % PHMB are commonly used in wound antiseptics. Additionally, wound dressings containing PHMB have been shown to reduce bacterial burden and chronic wound pain in vivo (Eberlein et al., 2012).

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To clearly distinguish PHMB from PAPB, Substance differentiation. To clearly distinguish PHMB [200 % mg ml^{-1} (20 % aqueous solution); (C_{6}H_{14}N_{2}O); CAS no. 27083-27-8; Lonza] from PAPB [200 mg ml^{-1} (20 % aqueous solution); (C_{6}H_{14}N_{2}O); CAS 133029-32-0; Bonding], an ultra-high-performance liquid chromatography-tandem MS (UPLC/Q-TOF-MS) was performed by the Institute of Organic Chemistry at the Leibniz University of Hannover, Germany. Briefly, the components of the solutions PHMB and PAPB were separated using a C18 column, subsequently fragmented by collision and analysed by a quadrupole time-of-flight mass spectrometer. First, specific ions to distinguish the substances were identified. Subsequently, the identified specific ions were determined and quantified, and their chemical formulas were validated, to clearly distinguish PHMB from PAPB.

Preparation of substances. To determine the minimum bactericidal concentration (MBC), both substances were diluted with casein/soy peptone broth (CSB) to reach final concentrations between 0.005 % and 1.0 %. For PAPB, concentrations of 0.05 mg ml^{-1} (0.005 %), 0.1 mg ml^{-1} (0.01 %), 0.5 mg ml^{-1} (0.05 %) and 1 mg ml^{-1} (0.1 %; v/v) were also prepared with 0.5 % w/v sodium chloride for efficacy verification via a quantitative suspension method.

The tested dressings consisted of a hydrophilic, absorbent layer (polyurethane foam) coated with a polyurethane foil, containing either 0.5 % PHMB or PAPB [DracoFoam PHMB; DracoFoam with PAPB (sample dressing); Dr. Ausbüttel & Co.].

For the evaluation of cytotoxicity, the substances were diluted with Dulbecco’s modified eagle medium (DMEM) high Glc (Biochrom) for human keratinocytes (HaCaTs) or Roswell Park Memorial Institute medium (RPMI)-1640 (Biochrom) for murine fibroblasts (L929), for human keratinocytes (HaCaTs) or Roswell Park Memorial Institute medium (RPMI)-1640 (Biochrom) for murine fibroblasts (L929), for murine fibroblasts (L929). For final concentrations ranging from 0.05 mg ml^{-1} (0.005 %) to 10 mg ml^{-1} (1.0 %, v/v). Additionally, in the case of PAPB, higher concentrations ranging from 2.5 mg ml^{-1} (0.25 %) to 30 mg ml^{-1} (3.0 %, v/v) were prepared in the same manner to test an extended range of the substance.

Cell culture. Human keratinocytes (HaCaTs) and murine fibroblasts (L929) (LGC Standards) were used for in vitro cytotoxicity tests. Cells were cultivated in DMEM high Glc for HaCaTs or RPMI 1640 for L929, supplemented with 10 % fetal bovine serum (Biochrom) in 5 % CO_{2} at 37 °C for 48 h.

Test organisms and nutrient solutions. For susceptibility testing, the following bacterial strains were used: S. aureus (DSM-799), P. aeruginosa (DSM-939) and E. coli (DSM-11250; all DSMZ). All of them
were cultivated on casein/soy peptone agar (15 mg ml\(^{-1}\) agar in CSB; AppliChem).

For each bacterial strain, test suspensions were prepared from one fresh colony each in CSB containing 15 mg ml\(^{-1}\) casein peptone, 5 mg ml\(^{-1}\) soy peptone, 5 mg ml\(^{-1}\) sodium chloride and 50 ml aqua bidest. The pH value was adjusted to 7.2 using 5 M sodium hydroxide (AppliChem) and incubated overnight at 37 °C under aerobic conditions. For both suspension and foam investigations, initial bacterial colony-forming unit (CFU) counts (-1×10\(^8\) ml\(^{-1}\)) were determined by spreading untreated control cultures of each experiment onto agar plates, allowing exact calculations of surviving organisms as well as of reduction rates.

**Cytotoxicity of PHMB and PAPB in vitro.** Cytotoxicity of PHMB and PAPB was determined based on DIN EN ISO 10993-5 (ISO, 2009). Briefly, cells were trypsinized (Trypsin-EDTA; Biochrom), counted (NucleoCounter; ChemoMetec) and diluted to 1×10\(^5\) cells ml\(^{-1}\). For tests, 100 µl of the cell suspension was placed in 96-well microtitre plates (Sarstedt) and cultivated for 24 h in 5 % CO\(_2\) at 37 °C. Afterwards, cell culture medium was replaced by the different test concentrations of either PHMB or PAPB and once again incubated under the described conditions. The percentage of surviving cells was detected via XTT assay (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; AppliChem) after 24 and 72 h of incubation according to the manufacturers’ instructions, and photometrically determined at 600 and 480 nm.

**Antiseptic efficacy of PHMB and PAPB in vitro.** MBCs, defined as the lowest concentration of an antibacterial agent reducing the viability of the initial bacterial inoculum by ≥99.9 %, were determined by the method of micro-bouillon dilution in accordance with DIN 58940-7 (DIN, 2009) to test the susceptibility of the three test organisms against PHMB and PAPB. Briefly, 50 µl of prepared concentrations (0.1–20 mg ml\(^{-1}\); 0.01–2.0 %, v/v) of the substances was added to the wells of a 96-well microtitre plate (Sarstedt) and subsequently inoculated with 50 µl of the bacterial test suspension, which had been adjusted to 2–8×10\(^5\) CFUs ml\(^{-1}\), resulting in the above-described final test concentrations of PHMB and PAPB ranging from 0.05 to 10 mg ml\(^{-1}\) (0.005 to 1.0 %, v/v). After 6 and 24 h of incubation at 37 °C under aerobic conditions for PHMB and 6, 24, 48 and 72 h for PAPB, the OD was measured via a laser-based micro-plate nephelometer (Nephelostar; BMG Labtech), and aliquots were spread onto nutrient agar plates in triplicate. After another 24 h of incubation under the same conditions, bacterial counts (CFU ml\(^{-1}\)) were carried out with a Colony Counter Pen (eCountTM, VWR), and MBCs were determined.

A quantitative suspension method based on DIN EN 13727 (DIN, 2012) was adapted and modified to test PAPB solutions in four different concentrations [0.05 mg ml\(^{-1}\) (0.005 %), 0.1 mg ml\(^{-1}\) (0.01 %), 0.5 mg ml\(^{-1}\) (0.05 %) and 1 mg ml\(^{-1}\) (0.1 %, v/v)] with respect to their antiseptic efficacy against the three test organisms without organic load. Briefly, serial 10-fold dilutions of the bacterial suspension were prepared in CSB and inoculated with PAPB solutions to result in the desired final concentrations. The resulting solutions were incubated for 0.5, 1 or 2 h at room temperature. Afterwards, aliquots (100 µl) were spread onto nutrient agar plates and incubated at 37 °C overnight under aerobic conditions. Each analysis was performed in triplicate, and surviving bacteria (CFU ml\(^{-1}\)) were counted with a Colony Counter Pen.

To evaluate the antiseptic efficacy of the substances embedded in a wound dressing, two polyurethane foams containing either PHMB or PAPB were tested in a modified time-kill assay based on ISO 20743 (ISO, 2012). The purpose of these tests was to investigate the efficacy of the substances in a dressing formulation, therefore imitating the clinical
treatment situation of infected exuding wounds. For the modified time-
kill assay, ten 1 cm² pieces of each dressing were prepared and placed in
multi-well plates (Sarstedt) in an aseptic manner. A bacterial test sus-
pension, prepared as previously described, was then serially 10-fold
plated in CSB, and 100 µl of each dilution step was added to the
appropriate well containing a specimen. Also, a control of the untreated
dressing. Afterwards, the dressings were incubated at 37
°C for a total of 72 h. At 2, 4, 6, 24, 48 and 72 h, aliquots of
50 µl of each dilution step were obtained by stamping out the specimen
again three times in the same manner and spread onto nutrient agar
plates. Surviving bacterial counts (CFU ml⁻¹) were carried out after
overnight incubation at 37°C with a Colony Counter Pen. All experi-
ments were performed in triplicate.

Statistical analysis. The evaluation of cytotoxicity was performed
according to the established cytotoxicity scale of the Fraunhofer Insti-
tute for Interfacial Engineering and Biotechnology (fG). In this scale,
referring to the positive control as being 100 %, the survival rate of pro-
liferating cells is ranked. A proliferation rate over 80 % is termed non-
cytotoxic, whilst a value below 61 % is considered highly cytotoxic.

Bactericidal activity was indicated by a reduction of bacterial counts in
log₁₀ CFU ml⁻¹ and defined as none for a <0.5, low for a 0.5–1, moder-
ate for a >1 to ≤3 and high for a >3 log reduction, based on previously
used assessment scores (Wiegand et al., 2009a).

Mean values and SD were calculated from triplicates. Differences were
considered statistically significant at P<0.05. In vitro cytotoxicity and
antiseptic effects of PHMB and PAPB were assessed by unpaired t-test.
Comparative statistical evaluations of the antimicrobial efficacy of the
two substances were performed using paired t-test. The statistics package
SPSS Statistics (IBM) was used for statistical analysis.

RESULTS

Substance differentiation via UPLC/Q-TOF-MS

Chromatography of PHMB and PAPB revealed their chemical
difference based on specific fragments due to the earlier elution of PAPB fragments compared to the less polar
PHMB fragments whilst passing through the C18 column (Fig. 2). Additionally, specific ions expected for differentiation
of each substance could be verified by identifying their molecular formula based on the exact mass to charge ratio
and determined via high-resolution MS (Fig. 1c–f).

Cytotoxicity of PHMB and PAPB

In all tested concentrations, PHMB showed a high cyto-
xicity against human HaCaT and L929 murine fibroblast cell
lines after 24 and 72 h of incubation, never exceeding a sur-
vival rate of 27 %. PAPB displayed significantly lower cyto-
xicity in concentrations ranging from 0.05 mg ml⁻¹
(0.005 %) to 1 mg ml⁻¹ (0.1 %, v/v). In concentrations up
to 1 mg ml⁻¹ (0.1 %), no cytotoxic effect could be detected
for L929 cell lines after 24 h, whereas for HaCaTs, a moder-
ate and high cytotoxicity occurred for 0.5 mg ml⁻¹ (0.05 %)
and 1 mg ml⁻¹ (0.1 %) PAPB (Fig. 3a). After 72 h, only a
weak cytotoxic effect on L929 of 0.5 mg ml⁻¹ (0.05 %) and
1 mg ml⁻¹ (0.1 %) PAPB could be observed, whilst for HaCaT
concentrations, up to 1 mg ml⁻¹ (0.1 %) can be classified as non-cytotoxic (Fig. 3b). However,
concentrations of $\geq 2.5 \text{ mg ml}^{-1}$ (0.25 %) PAPB revealed high cytotoxicity for both cell lines already after 24 h (Fig. 4). When directly compared, PAPB consistently showed a significantly higher cell survival rate than PHMB, irrespective of concentration and incubation time ($P \leq 0.0006$; Fig. 3a, b).

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**Fig. 3.** Cytotoxicity test for PHMB and PAPB (0.05–1 mg ml$^{-1}$; 0.005–0.1 %) according to DIN EN ISO 10993-5 on L929 murine fibroblasts and human HaCaT cell lines after 24 h (a) and 72 h (b) of incubation. Visualization as cell survival rate (%) according to the cytotoxicity scale of the Fraunhofer Institute for Interfacial Engineering and Biotechnology, IGB, Stuttgart, Germany [proliferation rate >81 % (light line) = non-cytotoxic; rate <61 % (dark line) = highly cytotoxic]. The positive control is stated as 100 %. Values are expressed as mean±SD with significant differences indicated as *$P<0.05$, **$P<0.01$ and ***$P<0.001$. 

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MBCs of PHMB and PAPB

With an MBC<sub>6h</sub> of <0.05 mg ml<sup>-1</sup> (0.005 %), therefore eliminating 99.9 % of each bacterial test strain after only 6 h in the lowest tested concentration, PHMB displayed a very high efficacy (Table. 1), whereas for PAPB, the MBCs were significantly higher. Whilst the MBC<sub>24h</sub> for <i>S. aureus</i> was 2.5 mg ml<sup>-1</sup> (0.25 %), double the concentration was needed for <i>E. coli</i> (5 mg ml<sup>-1</sup>; 0.5 %) and again twice as much for <i>P. aeruginosa</i> (10 mg ml<sup>-1</sup>; 1.0 %). After 48 h, the MBC<sub>48h</sub> of <i>S. aureus</i> and <i>P. aeruginosa</i> remained unchanged, whilst it dropped to 0.5 mg ml<sup>-1</sup> (0.05 %) for <i>E. coli</i>. No further changes were detected for MBC<sub>72h</sub>.

Antimicrobial efficacy of PAPB solution

All tested concentrations of PAPB showed no reduction in bacterial counts at any tested time in the quantitative suspension method. Neither <i>S. aureus</i> nor <i>E. coli</i> or <i>P. aeruginosa</i> exceeded a maximum log reduction of 0.16 after 2 h at the highest tested concentration (1 mg ml<sup>-1</sup>; 0.1 %).

Antimicrobial efficacy of foam dressings

As an antiseptic dressing formulation, the foam containing 0.5 % PHMB exhibited a high antiseptic efficacy against <i>S. aureus</i> and <i>E. coli</i>, whilst none could be detected for <i>P.

Table 1. MBC determination after 6 and 24 h for PHMB and after 6, 24, 48 and 72 h for PAPB against <i>S. aureus</i>, <i>E. coli</i> and <i>P. aeruginosa</i> tested according to DIN 58940-7 (range 0.05–10 mg ml<sup>-1</sup>; 0.005 %–1.0 %). Values are expressed in mg ml<sup>-1</sup>.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>PHMB MBC (in mg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>PAPB MBC (in mg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
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<tr>
<td></td>
<td>MBC&lt;sub&gt;6h&lt;/sub&gt;</td>
<td>MBC&lt;sub&gt;24h&lt;/sub&gt;</td>
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<td>&lt;i&gt;S. aureus&lt;/i&gt;</td>
<td>&lt;0.05</td>
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<td>&lt;i&gt;E. coli&lt;/i&gt;</td>
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<td>&lt;i&gt;P. aeruginosa&lt;/i&gt;</td>
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aeruginosa (Fig. 5). For S. aureus and E. coli, reductions of 3.52±0.67 (P=0.012) and 3.56±0.28 (P=0.002), respectively, could be observed after only 4 h of exposure for the PHMB foam, increasing to 5.86±0.44 (P=0.002) and 4.09±0.35 (P=0.002) after 72 h, therefore displaying a high bactericidal activity. Nevertheless, the PHMB foam failed to completely eradicate the two bacteria at any time. For P. aeruginosa, an initial moderate bactericidal activity of 1.42±0.29 (P=0.093) could be observed after 2 h, but bacterial counts increased again over time, and after 72 h, maximum evaluable bacterial counts were obtained, indicating no overall antiseptic efficacy. As for the PAPB foam dressing, no reduction of bacterial counts could be observed for S. aureus, E. coli and P. aeruginosa within 72 h. Bacterial growth rates correlated with those of the control foam without incorporated antimicrobial substance and resulted in maximum evaluable bacterial counts for the three tested bacterial strains. Therefore, PHMB showed a significantly higher antiseptic efficacy than PAPB after 72 h for S. aureus and E. coli (P<0.0001).

**DISCUSSION**

Scientific as well as commercial labelling and differentiation of the substances PHMB and PAPB are often inaccurate. In different studies, the same product (ReNu Dymed; Bausch & Lomb) was stated to either contain PAPB (Sutton et al., 1991) or PHMB (Cano-Parra et al., 1999). Several, also recent, publications stated PAPB and PHMB to be identical (Burger et al., 1994; Leysen et al., 2014), whilst others use the abbreviation PHMB for polyaminopropyl biguanide and polyhexamethylene biguanide alike (Choy et al., 2013), implying the substances can be considered equal. In order to clearly distinguish PHMB and PAPB, a UPLC/Q-TOF-MS was performed for the present study based on different monomeric and dimeric by-products identifying the substances ( unpublished data, Dr Gerald Dräger, Institute for Organic Chemistry, Leibniz University Hannover, Germany) (Fig. 1c–f).

Considering the obtained results to be in line with the clear differentiation of the substances, the question arises whether the previously declared strong antiseptic efficacy of PAPB, reported in earlier works (Burger et al., 1994), does not actually need to be attributed to PHMB under the false assumption that the substances are equal. Referring to the presented results of the UPLC/Q-TOF-MS analysis, PHMB and PAPB show the same basic chemical structure consisting of biguanide groups connected by methylene chains, but with PAPB having shorter chain lengths, resulting in a relevant difference in chemical structure.

So far, little evidence of the efficacy of PAPB has been published. Contrary to earlier results (Lui et al., 2009), the present study demonstrated that, as a solution, only concentrations exceeding 2.5 mg ml⁻¹ (0.25%) had an acceptable bactericidal effect (MBC) within the first 24 h. The efficacy of the PHMB solution was shown to be 50-fold higher. Only by extending the time of exposure, was the efficacy of PAPB partially improved. However, this time-dependent antibacterial potential could not be confirmed in the case of its usage as an antiseptic substance or add-on in foam dressings. The overall efficacy of PAPB was similar to that of the control foam without antiseptic substance. It is still not known from this evaluation whether PAPB might show an additional reduction in antibacterial activity against S. aureus, including Methicillin-resistant Staphylococcus aureu (MRSA), in the presence of albumin, as observed for PHMB (Kapalschinski et al., 2013).

Even though PAPB and PHMB are of similar chemical structure, their specific differences need to be emphasized, in particular with regard to their proposed antibacterial mode of action. Both belong to the group of cationic antimicrobial agents. They are polymeric biguanides, consisting of polycationic linear polymers comprising a hydrophobic backbone with multiple cationic groupings separated by methylene chains (Gilbert et al., 2005). These polymeric biguanides are believed to exert their antibacterial effect by rapidly binding to the negatively charged cell membrane of Gram-positive and Gram-negative bacteria via multiple positively charged domains and bridging acidic phospholipids through the hydrophobic methylene groups (Broxton et al., 1984a; Ikeda et al., 1985a, b). Earlier research showed that with increasing polymer chain length, the antimicrobial activity increased as well (Broxton et al., 1983). Because of the polymeric nature of PHMB, a sequestration of the acidic phospholipids into domains of the same phospholipid types occurs, leading to membrane dysfunction and total loss of the permeability barrier over time resulting in increased fluidity, loss of integration, cellular destruction and transition of PHMB into the cell (Broxton et al., 1983, 1984a, b, c; Ikeda et al., 1985a). A main difference between PAPB and PHMB is the length of their methylene chains: PHMB generally consists of six methylene groups (hexamethylene) which is especially favourable for the total disruption of the bacterial cell membrane (Kaehn, 2010; Ikeda et al., 1985b), whilst PAPB contains shorter methylene chains (Fig. 1a, b). This results in higher polarity of PAPB, further complicating the interaction with the negatively charged bacterial lipid bilayer as well as its penetration. In contrast, PHMB contains three additional carbon atoms resulting in a longer polymer chain length and lower polarity making it more lipophilic than PAPB. Because of these attributes PHMB displays increased membrane permeability, facilitating its interaction with bacterial membranes compared to PAPB and its transfer into the cell, where it disturbs the metabolism of the micro-organisms [reviewed by Hubner & Kramer (2010) and Kaehn (2010)]. Recent research further supports the interaction with bacterial membranes as the mode of action for PHMB, stating that it disturbs the first layer of the membrane lipid bilayer (Chadeau et al., 2012) by electrostatic as well as hydrophobic interactions and dehydration (Souza et al., 2015). However, a new study also strengthened the hypothesis of additional mechanisms of the bactericidal effect of PHMB based on the interaction of the infiltrated substance with bacterial chromosomes (Chindera et al., 2016). In this work, PHMB was shown to enter bacterial cells and condense chromosomes as well as
arrest cell division, ultimately leading to the death of treated bacteria. The inferior bactericidal effect of PAPB can, therefore, be assumed to rely on its disadvantageous chemical structure with shorter methylene chains. Not only its ability to interact and disrupt bacterial membranes is reduced, but also its higher polarity cell entry is decreased, preventing condensation of chromosomes and disruption of bacterial metabolism. These assumptions are consistent with the results of our study and would explain the lack of antiseptic efficacy of PAPB compared to the highly efficient PHMB.

In contrast to the effects on bacterial cell membranes, neutral phospholipids found in the human cell envelope are only marginally influenced by PHMB (Broxton et al., 1984b; Ikeda et al., 1983). Also, Chindera et al. (2016) demonstrated that despite an uptake of PHMB into several mammalian cell lines, no transition into nuclei occurs owing to entrapment of PHMB in endosomes. As a consequence, PHMB displays an excellent combination of bactericidal efficacy and low cytotoxicity to mammalian cells. The presented results show that PAPB possesses similar, low to non-cytotoxic effects on fibroblasts and keratinocytes, presumably owing to its similar chemical structure. Regarding its cytotoxicity, PAPB seems to be even less cytotoxic than PHMB at comparable concentrations. This effect may also be accounted for by the shorter methylene chains of PAPB, which decrease the interaction with mammalian cell membranes and cell entry. Unfortunately, significantly higher doses of PAPB (>5 mg ml⁻¹; 0.5 %) were required to show an effect on bacteria, and at concentrations, the cytotoxicity corresponds to that of PHMB. Thus, PAPB showed

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**Fig. 5.** Modified time-kill assay of foam dressings containing 0.5 % PHMB or PAPB against *S. aureus*, *E. coli* and *P. aeruginosa* after 2, 4, 6, 24, 48 and 72 h. Values are expressed in $\Delta \log_{10}$ CFU ml⁻¹ over time with significant differences indicated as *$P<0.05$, **$P<0.01$ and ***$P<0.001$. $\Delta \log_{10}$ CFU ml⁻¹ is the difference in $\log_{10}$ CFU ml⁻¹ of the respective time intervals to the initial bacterial count ($t=0$). Strains: (a) *S. aureus* DSM-799, (b) *E. coli* DSM-11250, (c) *P. aeruginosa* DSM-939.
a low toxicity on mammalian cells, but not the necessary antibacterial effect to be of advantage.

In the past few years, discussion about a possible carcinogenic potential of PHMB has recurred. Early studies suggesting PHMB caused haemangiosarcomas of the liver or other organs (Clapp et al., 1977a, b) led to the recent re-evaluation of PHMB by the RAC of the European Chemicals Agency (ECHA). Although these studies were only performed in mice with doses of PHMB much higher than in clinical use (750 mg kg⁻¹ KG and 856 mg kg⁻¹ KG), PHMB was classified as carcinogenic, and named Carc 2–H351 (CLP) based on these findings. However, the statement was limited by the awareness that the overall evidence on the carcinogenic potential of PHMB is disputable. The Scientific Committee on Consumer Safety recently published an opinion on PHMB (SCCS, 2014). Therein, PHMB is stated to have no genotoxic potential in vivo, whilst in terms of mutagenicity, the opinion of the ECHA’s RAC was adopted. In contrast, in 2004, the US Environmental Protection Agency evaluated PHMB as displaying no toxicity, no mutagenicity and carcinogenicity (reviewed by Hubner & Kramer, 2010). In accord with the US Environmental Protection Agency, the Australian Pesticides and Veterinary Medicines Authority also stated in 2011 that PHMB showed no genotoxicity and would not take the assumed carcinogenicity into consideration to discontinue the registration of products containing PHMB (APVMA, 2011). Additionally, no further studies since those reported by Clapp et al. (1977a, b) and Milburn et al. (1996) have shown a mutagenic potential for PHMB. A more recent study actually estimates that PHMB does not exhibit clear and remarkable epigenetic properties (Creppy et al., 2014).

To the authors’ knowledge, no studies regarding the carcinogenicity of PAPB exist. PAPB belongs to the biguanide family, like PHMB and dimethylbiguanide (DMB; Metformin®), an established antidiabetic drug. Neither for PHMB (Kaehn, 2010) nor for DMB, have toxicity, genotoxicity or mutagenicity been observed in preclinical studies or conclusively proven. On the contrary, DMB is even associated with a lower risk of developing colon cancer in patients with diabetes mellitus type II (Libby et al., 1996) have shown a mutagenic potential for PHMB. A more recent study actually estimates that PHMB does not exhibit clear and remarkable epigenetic properties (Creppy et al., 2014).

The results on differentiation, antiseptic efficacy and cytotoxicity on mammalian cells of PHMB and PAPB in vitro presented here add valuable data to the profile of these agents. It should especially be pointed out that PHMB and PAPB showed remarkable differences in performance in vitro. This emphasizes the difference of the two substances despite their chemical similarities and should raise doubts about the correctness of considering PHMB and PAPB as equal. Nevertheless, work still needs to be done to further extend the knowledge on the investigated substances. A limitation to this study is the theoretical character of understanding the mode of action of PAPB at this moment. Further experiments such as those for PHMB are needed to verify the exact reasons for the inferior bactericidal efficacy of PAPB. Also, generally further insight into the toxicity profile of different polymeric biguanides as cationic antimicrobial agents is needed to resolve ongoing debates about mutagenicity, carcinogenicity and toxicity to host cells.

In summary, PAPB showed lower cytotoxicity to mammalian cells than PHMB at comparable concentrations, but no antiseptic effect could be observed. Therefore, it could be presumed that the beneficial microbical effects of these substances come with a certain cytotoxic potential. Even though PAPB and PHMB are highly similar in their chemical structure, slight differences result in a strong discrepancy in bactericidal activity. The results reveal PHMB to be clearly superior to PAPB in terms of antiseptic efficacy. This emphasizes the need to thoroughly evaluate potential new antiseptic formulations and accurately distinguish between close chemical relatives such as PHMB and PAPB.

ACKNOWLEDGEMENTS

We thank Gerald Drüger of the Institute for Organic Chemistry of the Leibniz University Hannover, Germany, for the chemical distinction of the PHMB and PAPB solutions and the Thuringian Institute for Textile and Plastics Research, Rudolstadt, Germany, for the determination of cytotoxicity. Also, the authors thank A. Richard for her technical support. Source of Funding: This work was supported by grants from Dr. Ausbüttel & Co. GmbH, Witten, Germany. The authors declare that no competing interests exist and that there is no conflict of interest.

REFERENCES


DIN (2009). EN 58940-7:2009-08 - Medical microbiology. Susceptibility testing of microbial pathogens to antimicrobial agents - Part 7: Determination of the minimum bactericidal concentration (MBC) with the method of microbouillon dilution. (Text in German and English).
DIN (2012). EN 13727:2012+A1:2013 - Chemical disinfectants and antisepsics. Test method and requirements (phase 2, step 1) - Poly(hexamethylene) biguanidehydrochloride or PHMB.