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Biocompatibility and Antibacterial Effects of 6-Deoxy-6-Aminoethyleneamino Cellulose

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Abstract

Recently, there is a need of alternatives to antibiotics due to increasing antibiotic-resistant microorganism. Promising classes of bioactive polymers are 6-deoxy-6-amino cellulose derivatives. The purpose of the study was the assessment of the biocompatibility of 6-deoxy-6-aminoethyleneamino cellulose (AEAC) with different degree of substitution (DS). HaCaT keratinocyte cell viability was analyzed by measuring the cellular ATP content. The antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* was examined by microplate laser nephelometry. Thus, the ratio of half-maximal lethal concentration (LC_{50}) and half-maximal inhibitory concentration (IC_{50}) was calculated and described as biocompatibility index. The study revealed that biocompatibility of AEAC depends on the DS. AEAC of low DS (0.3) showed the best biocompatibility.

Keywords

6-Deoxy-6-Aminoethyleneamino Celluloses, Antibacterial, Bacteria, Biocompatibility, Biomacromolecule, Keratinocyte

1. Introduction

There are different ways to deal with bacterial infections. To prevent or to treat bacterial infections antiseptics, disinfectants, antimicrobial peptides, or antibiotics are the means of choice. However, the latter are intensely discussed because frequent use of antibiotics leads to an increase of antibiotic resistance in bacteria. Thus, there is a need for alternatives. Antimicrobial polymers are considered to be valid options for commonly used products [1] [2] [3] [4] [5]. A choice could

be the use of chemically modified cellulose such as 6-deoxy-6-amino cellulose derivatives. A typical example of this novel class of cellulose derivatives is 6-deoxy-6-aminoethyleneamino cellulose (AEAC) [3] [4] [6]. AEAC is described to be biocompatible [2] and hemocompatible [7].

AEACs are synthesized by nucleophilic displacement reaction of p-toluenesulfonic acid ester of cellulose with ethylene diamine [4] [7] [8] introducing amino moieties into the cellulose backbone that are water-soluble and may be positively charged depending on the pH value of the system [4]. The number of functional groups generated in the cellulose derivative is described as degree of substitution (DS) [9]. It is proposed that the functionalization with amino groups leads to antimicrobial activity. Besides antimicrobial efficacy, cytocompatibility is most important for the use of biomaterials in medical applications. Biocompatibility has become the central request for the medical application of materials and devices [10] [11] [12] [13]. Müller and Kramer defined a biocompatibility index (BI) for antiseptics based on the damage of murine fibroblasts and the MIC values for *S. aureus* and *E. coli* [14]. Following their approach [14], the BI was used to rate antibacterial activity of AEAC and effects on human cells. Here, the BI was defined as the ratio of the half-maximal lethal concentration (LC_{50}) and the half-maximal inhibitory concentration (IC_{50}). Accordingly, a BI greater than 1 will describe a substance exhibiting a high antimicrobial activity combined with a relatively low cytotoxicity (**Figure 1(a)**), whereas a BI less than 1 will indicate an antimicrobial effective sample with a relatively high cytotoxicity (**Figure 1(b)**).

The aim of this study was the assessment of biocompatibility of AEAC with different DS. With regard to cytocompatibility, HaCaT keratinocytes have been used as a model system to determine cell viability by measuring cellular ATP content. *S. aureus* and *K. pneumoniae* were used as model organisms to investigate antibacterial activity of AEAC using microplate laser nephelometry (MLN). The ratio of half-maximal lethal concentration (LC_{50}) and half-maximal inhibitory

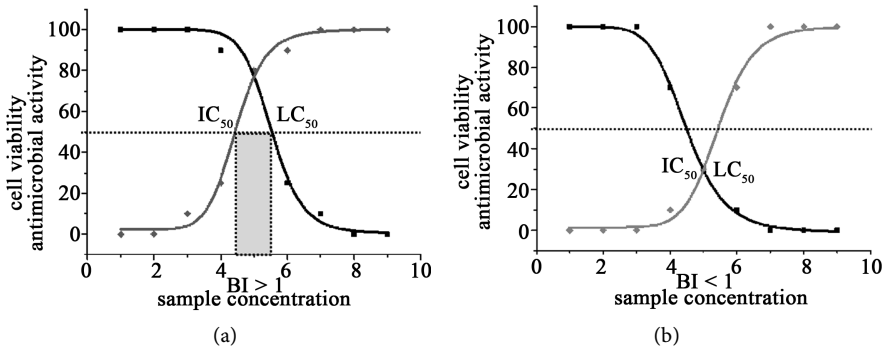

Figure 1. The biocompatibility index (BI) is composed of the ratio of half maximal lethal concentration (LC_{50}) to half maximal inhibitory concentration (IC_{50}). A BI greater than 1 indicates a substance with a high antimicrobial activity and good cell compatibility. In contrast a BI less than 1 indicates indeed a high antimicrobial activity but more than 50% of the human cells are death. The dotted line marks cell viability or antimicrobial activity at 50%.

concentration (IC_{50}) was calculated and described as biocompatibility index (BI). In addition, we determined the effect of DS on cytocompatibility and antimicrobial activity.

2. Materials and Methods

2.1. Synthesis of 6-Deoxy-6-Aminoethyleneamino Cellulose

Water-soluble 6-deoxy-6-aminoethyleneamino cellulose (AEAC, Figure 2) was synthesized by nucleophilic displacement reaction of cellulose tosylate as described elsewhere [2] [7]. AEAC with different degree of substitution (DS) of 0.3, 0.5, 0.7, and 0.9 were prepared. The initial AEAC concentration was 10 mg/mL. Serial AEAC dilutions were prepared with respective cultivation medium.

2.2. Cultivation of HaCaT Keratinocytes

Human HaCaT keratinocytes (kindly provided by N. E. Fusenig, German Cancer Research Center Heidelberg, Germany) were cultured at 37˚C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (Promocell, Germany) supplemented with 1% antibiotic-antimycotic solution (Promocell, Germany) and 10% fetal bovine serum (Promocell, Germany) as described elsewhere [15].

2.3. Determination of Cell Viability

HaCaT keratinocytes were incubated with AEAC dilutions for 24 h and afterwards cell viability was determined using a luminometric adenosine triphosphate (ATP) assay (ATPLite Kit; Perkin Elmer Life Sciences, Belgium) according to the manufacturer's instructions. The ATP-dependent light generation was measured using a microplate luminometer (LUMIstar Galaxy; BMG Labtech, Germany). The total amount of cellular ATP was measured after the cells were lysed. ATP concentration, calculated on the basis of a standard curve, is proportional to the number of metabolic active cells. Cell numbers were therefore calculated according to an ATP-cell number-standard curve [15]. Cells cultured in medium alone served as negative control. Triton-X100 (0.01%) was used as a positive control for cytotoxicity. For evaluation, cellular proliferation under test conditions was expressed as percentage of the negative control. Half-maximal lethal concentration (LC_{50}) was calculated. Each substance concentration was

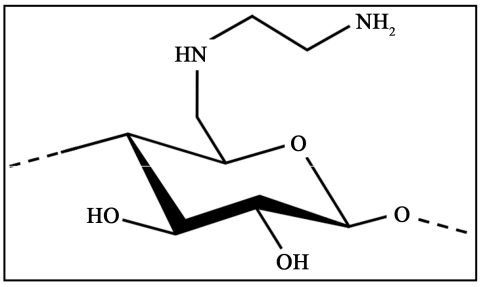

Figure 2. Schematic representation of 6-deoxy-6-aminoethyleneamino cellulose.

tested in eightfold, and all experiments were performed at least twice.

2.4. Cultivation of Bacteria

Staphylococcus aureus ATCC 6538 and *Klebsiella pneumoniae* ATCC 4352 were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). Strain maintenance was performed on Columbia agar plates with 5% sheep blood (Biomeriéux, France). Bacterial suspensions were prepared in CASO medium (Oxoid, UK) according to Finger *et al.* [16].

2.5. Determination of Antibacterial Activity

Effects of AEAC on *S. aureus* and *K. pneumoniae* were determined by microplate laser nephelometry (MLN) as previously reported [16] [17] [18] [19]. For this, bacterial suspensions ((5×10^2 - 7×10^2 colony forming units) were incubated with AEAC dilutions in a microplate laser nephelometer (NEPHELOstar Galaxy; BMG Labtech, Germany) for 24 h at 37˚C. CASO medium alone was used as negative control and Chlorhexidine digluconate (0.05%) as a positive control for antibacterial activity. The half-maximal inhibitory concentration (IC_{50}) of the AEAC under the experimental conditions used was calculated from the growth curves over 24 h.

2.6. Data Analysis and Statistics

Experiments and measurements were performed in duplicate each. All values are expressed as means ± SE. LC_{50} and IC_{50} values were calculated using a logistic fit function as described elsewhere [5]

$$y = \frac{A_2 + (A_1 - A_2)}{\left(1 + \left[\frac{x}{x_0}\right]^p\right)}$$

with A_1 = upper limit, A_2 = lower limit, x_0 = LC_{50} or IC_{50}, p = slope of the curve; Origin 7.5, USA.

The BI was calculated from the LC_{50} and IC_{50} values obtained after 24 h.

$$\mathrm{BI} = \frac{\mathrm{LC}_{50}}{\mathrm{IC}_{50}}$$

3. Results

3.1. Effects of AEAC on HaCaT Keratinocyte's Cell Viability

For assessment of AEAC with regard to its effect on viability of HaCaT keratinocytes, cellular ATP content was determined using a luminometric assay. Because ATP can only be determined in living cells, information on cell viability is obtained. Hence, HaCaT keratinocytes were incubated with dissolved AEAC of different DS over 24 h. In general, the AEAC of different DS were compatible to the HaCaT cells. However, LC_{50} values showed a distinct tendency; cytocompatibility decreased with increasing DS. The AEAC with the low DS of 0.3 showed

the highest cytocompatibility, whereas the AEAC with the highest DS (0.9) was less cytocompatible (Table 1). Figure 3 shows the dependence of HaCaT cell viability on concentration and DS of the AEAC samples. Already a concentration of 39 µg/mL showed a distinct difference on HaCaT cell viability. AEAC with DS higher 0.5 exhibited a reduction in cell viability of 33% (AEAC-DS0.7) or 38% (AEAC-DS0.9) compared to the control. In case of AEAC with DS of 0.7, a distinct decline in cell viability was observed at a concentration of 625 µg/mL. For the AEAC with low DS of 0.3, cell viability is influenced at concentration higher than 2500 µg/mL (data not shown).

3.2. Antibacterial Activity of AEAC

The growth of *S. aureus* and *K. pneumoniae* under the influence of AEAC was monitored by MLN (Figure 4). It was found that the AEAC had bactericidal effects. It was shown that the antibacterial activity of AEAC depended on the DS. The IC_{50} values for both bacteria (Table 1) illustrate an increase of antibacterial

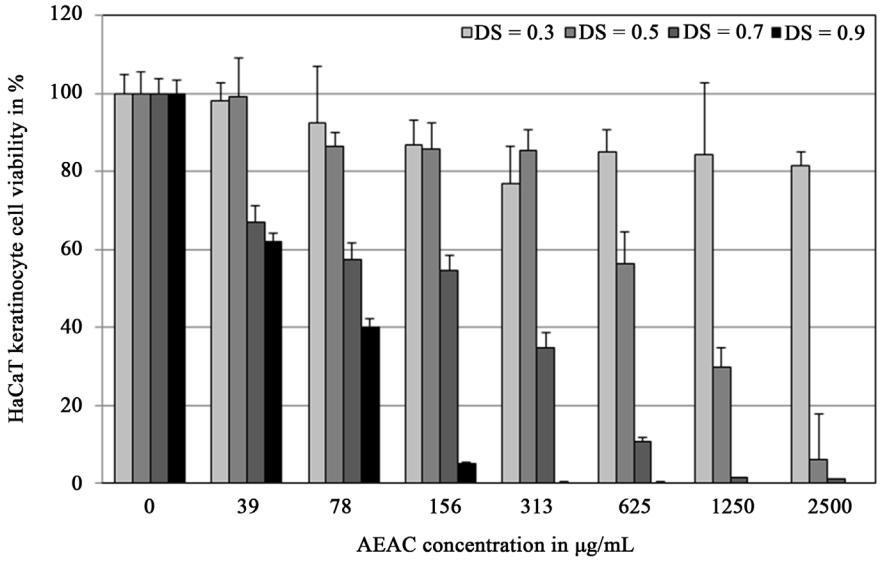

Figure 3. Determination of cell viability by measurement of the cellular ATP content after 24 h incubation of HaCaT keratinocytes with the different AEAC. AEAC exhibited concentration- and DS-dependent effects on HaCaT keratinocytes.

Table 1. LC_{50} and IC_{50} values were calculated by means of cell viability measurements and growth curves. The biocompatibility index (BI) was calculated by means of the ratio of LC_{50} and IC_{50} values.

DS_{AEAC}	LC_{50} in µg/mL HaCaT cells	IC_{50} in µg/mL *S. aureus*	BI (LC_{50}/IC_{50}) *S. aureus*	IC_{50} in µg/mL *K. pneumoniae*	BI (LC_{50}/IC_{50}) *K. pneumoniae*
0.3	3916.22 ± 35.04	90.21 ± 7.93	43	83.00 ± 1.27	47
0.5	858.29 ± 8.78	42.36 ± 0.79	20	192.17 ± 7.59	4
0.7	197.71 ± 33.64	17.91 ± 2.41	11	132.79 ± 17.66	1
0.9	58.87 ± 2.02	20.60 ± 0.29	3	60.95 ± 6.43	1

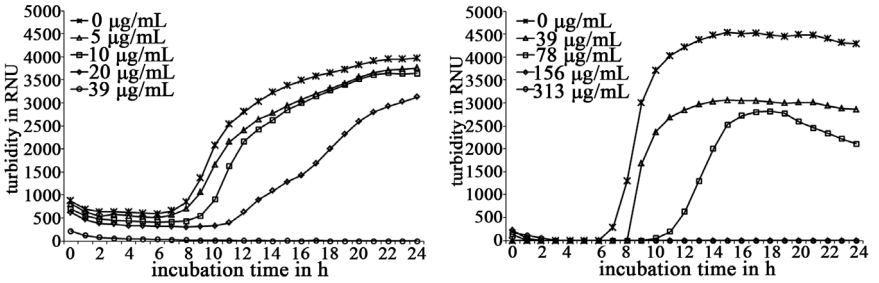

Figure 4. Growth curves determined by MLN over 24 h. Effects of AEAC-DS0.9 on *S. aureus* (a) and *K. pneumoniae* (b).

activity with increasing DS with the exception of AEAC with DS 0.3 and *K. pneumoniae*. The AEAC with the highest DS of 0.9 showed the strongest antibacterial effects, while the AEAC with the lowest DS of 0.3 was less effective against the bacteria. In general, AEAC were more effective against *S. aureus* compared to *K. pneumoniae*. For instance, 50% of *S. aureus* were inhibited with 20 µg/mL of AEAC with DS of 0.9, whereas threefold higher concentrations were needed to achieve the same effects on *K. pneumoniae*. In case of AEAC with DS of 0.7, the IC_{50} for *K. pneumoniae* (132.79 µg/mL) is almost seven times higher than for *S. aureus* (17.91 µg/mL). This trend was also observed for AEAC with DS of 0.5.

3.3. Biocompatibility of AEAC

The results of the cell viability assay and MLN were summarized as the ratio from LC_{50} to IC_{50} to determine the biocompatibility of AEAC. The value is referred to as biocompatibility index (BI). A BI greater than 1 indicates a substance with a high antimicrobial activity at the same concentration where more than 50% of the human cells are still viable *in vitro*, while, a BI less than 1 indicates a high antimicrobial activity, but a low cytocompatibility. This study demonstrated that the BI depends on the DS of AEAC. AEAC with the lowest DS of 0.3 was most compatible to HaCaT keratinocytes. The cytocompatibility decreases with increasing DS. This also applies to the determined BI (Table 1). Thus, the cytocompatibility for AEAC with low DS is better than for AEAC with higher DS (Figure 5). The highest BI from about 40 (*S. aureus*) and 50 (*K. pneumoniae*) could be calculated for AEAC with DS of 0.3 (Table 1). In addition, the BI determined with *S. aureus* was always higher than in connection with *K. pneumoniae* (Figure 5). Nonetheless, the BI was greater than or nearly 1 for every AEAC tested.

4. Discussion

Four AEAC with different DS were examined with regard to cytocompatibility and antibacterial activity. HaCaT keratinocytes were chosen to study the effects of AEAC on cell viability. This spontaneously immortalized cell line [20] is a

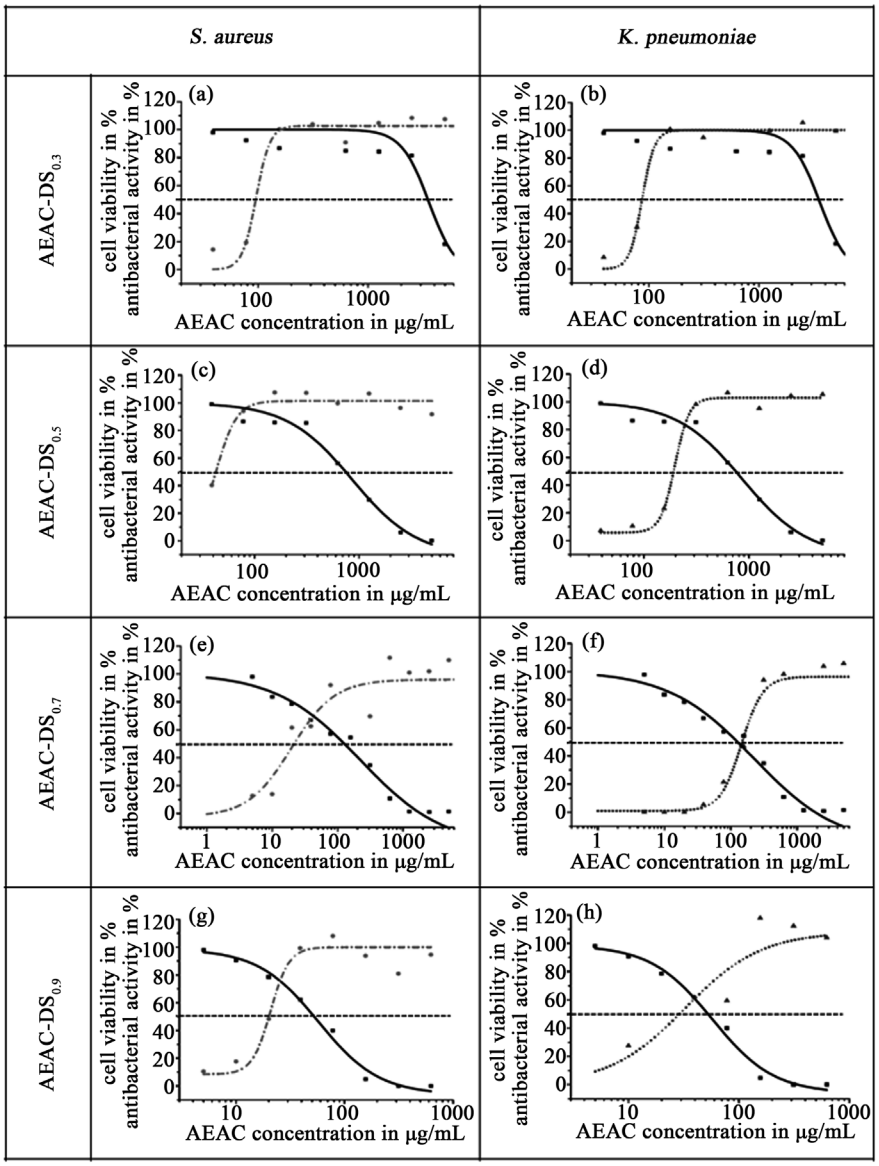

Figure 5. Effects of AEAC differ in DS on human HaCaT keratinocytes and on the bacteria *S. aureus* and *K. pneumoniae*. Graphs ((a)-(h)) demonstrate dose-response-curves for cell viability (presented in black) or antibacterial activity (presented in grey), while the dotted line marks 50% of cell viability or antibacterial activity. With increasing DS cell viability declined and antibacterial activity increased, except of AEAC-DS0.3 and *K. pneumoniae*.

suitable model to determine effects of diverse substances on cytology *in vitro* [10]. The antibacterial activity was monitored with respect to the bacteria *S. aureus* und *K. pneumoniae* by MLN. As described previously, MLN is a sensitive and suitable method to determine the influence of substances on microbial growth [17] [21] [22] [23]. To evaluate biocompatibility of AEAC, the ratio of LC_{50} and IC_{50} was calculated and referred to as biocompatibility index.

In consideration of the effects of AEAC on cell viability of HaCaT keratinocytes, it appears that the DS plays an important role. Thus, LC_{50} values decreased with

increasing DS. At low DS of AEAC, the effects on HaCaT keratinocytes were minimal. A decline in cell viability was observed with increasing DS. AEAC can be compared to chitosan to a certain extend; it appears to be a suitable synthetic version of chitosan. For chitosan it was reported to interact with negative charges in the skin [24]. He *et al.* found that chitosan and its derivatives could significantly change the secondary structure of keratin and increase the water content in Stratum corneum of skin, decrease HaCaT cell membrane potential and enhance cell membrane fluidity to various degrees [25]. The mode of action of chitosan leads to the suggestion that the positively charged AEAC substituents possibly interact with components of cell membranes or with components inside cells and this might lead to cytotoxic effects. However, the mode of action is not fully understood yet [26] [27]. The mode of action of AEAC suggested requires an examination of hemocompatibility for medical applications with tight contact to tissue and blood. Zieger *et al.* also tested AEAC with different DS in regard to hemolysis and markers for coagulation in human whole blood, human platelet rich plasma, human pooled plasma and erythrocytes suspensions [7]. It was described that AEAC showed concentration- and DS-dependent influence on hemocompatibility *in vitro*. Thus, AEAC with low DS had less influence on blood coagulation, a low hemolytic effect, and provided the highest hemocompatibility.

As for the biocompatibility of AEAC, it is crucial to determine antibacterial activity besides the cytocompatibility. The antimicrobial activity of chitosan was frequently mentioned in literature [19] [28] [29] [30] and could give some hints for understanding the effects of AEAC on microorganism. Hosseinnejad and Jafari reviewed different factors and mechanisms, which play a role in antimicrobial activity of chitosan [31]. For instance, molecular weight, degree of acetylation, concentration, and pH value as well as microbial species are named as antimicrobial properties.

In the present study, the differences of antibacterial efficacy observed against *S. aureus* and *K. pneumoniae* of AEAC possibly depend on the morphological composition of bacterial cell walls. AEACs were most effective against the Gram-positive bacterium *S. aureus*. For *K. pneumoniae*, a Gram-negative bacterium, higher AEAC concentrations were needed to inhibit bacterial growth compared to *S. aureus*. Hence, *S. aureus* was more sensitive to AEAC than *K. pneumoniae*. Jou also described comparable observation when *S. aureus* and *K. pneumoniae* were incubated with the chitosan derivative chitosan-N-hydroxy-2,3-propyl-N-methyl-N,N-diallylammonium methyl sulfate [28]. On the contrary to Gram-positive bacteria, Gram-negative bacteria possess a supplementary barrier in form of the lipopolysaccharide layer [16] [32] [33], which explains why Gram-negative are less sensitive.

In case of the AEAC with DS of 0.3, the determined IC_{50} values for both bacteria showed no distinctly difference in antibacterial activity (*S. aureus*: 90 µg/mL and *K. pneumoniae*: 83 µg/mL). A reason for this observation could be the fact that AEAC with low DS have the property to agglutinate surfaces [2] [34]. This

effect declines of samples of higher DS. Therefore, at DS higher 0.3 differences in antibacterial activity were observed with regard to cell wall composition. Similar to the antibacterial activity of AEAC with DS of 0.3, the BI for *S. aureus* and *K. pneumoniae* does not differ. Due to the sensitivity of *S. aureus* against AEAC, the BI are higher than for *K. pneumoniae*. With regard to the effects of AEAC on cell viability of HaCaT keratinocytes and on both bacteria, it is apparent that concentrations at which *S. aureus* was killed no effects on HaCaT cell viability were observed. That was the case for AEAC with DS of up to 0.5. AEAC with a DS above 0.5 showed only slight effects on cell viability by simultaneous killing of *S. aureus*. For *K. pneumoniae* this observation only applies to AEAC with DS of 0.3. With increasing DS, AEAC exhibited cytotoxic effects on HaCaT cells before growth of *K. pneumoniae* was inhibited completely that is also caused by less sensitivity of Gram-negative bacteria to antimicrobials as already mentioned.

It was found that AEAC with low DS (≤0.5) had less effect on cell viability of HaCaT keratinocytes than AEAC with higher DS (>0.5). Cell viability declined and antibacterial activity increased with increasing DS, except of AEAC-DS0.3 and *K. pneumoniae*. Whereat, *S. aureus* was more sensitive to the tested AEAC than *K. pneumoniae*.

AEAC with a maximum DS of 0.5 are most recommendable for medical applications because they showed the highest BI of all tested AEAC. Zieger *et al.* came to the same conclusion and recommend AEAC with DS of 0.5 for applications that require inert materials [7]. AEAC with low DS exhibited the lowest anticoagulant and hemolytic potential of AEAC tested. In addition to AEAC solutions, also applications of corresponding nanoparticles are possible [4] [6].

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