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BIOCOMPATIBILITY STUDIES

Original Research

Cytocompatibility testing of cyclodextrin-functionalized antimicrobial textiles—a comprehensive approach

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Abstract Functionalized textiles can be used in wound management to reduce the microbial burden in the wound area, to prevent wound infections, and to avoid cross-contamination between patients. In the present study, a comprehensive in vitro approach to enable the assessment of antibacterial activity of functionalized textiles and cytotoxicity of cyclodextrin (CD)-complexes with chlorhexidine diacetate (CHX), iodine (IOD), and polihexanide (PHMB) is suggested to evaluate their properties for supporting optimal conditions for wound healing. For all β-CD-antiseptic functionalized cotton samples a strong antibacterial effect on the Gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis* as well as on the Gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli* was proven. In addition, β-CD-CHX and β-CD-PHMB were effective against the yeast *Candida albicans*. The growth of *Pseudomonas aeruginosa* could be reduced significantly by β-CD-IOD and β-CD-PHMB. The established comprehensive testing system for determination of biocompatibility on human HaCaT keratinocytes is suitable for obtaining robust data on cell viability, cytotoxicity and mode of cell death of the β-CD-antiseptic-complexes. The promising results of the high antimicrobial activity of these functionalized textiles show the high potential of such materials in medical applications.

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1 Introduction

Current concepts in the area of health care and hygiene focus on the use of antiseptics rather than antibiotics. In the light of the increasing emergence of multi-resistant bacterial strains such as MRSA, due to the widespread application of systemic and topical antibiotics, the need for alternative antimicrobials is evident. A wide range of chemically different compounds are available for disinfecting purposes and, until recently, the most commonly used products in clinical settings were PVP-iodine, alcohols, acetic acid, hydrogen peroxide, sodium hypochlorite, silver nitrate, and silver sulfadiazil [1, 2]. However, detrimental effects on cutaneous cells due to these substances, e.g., silver, have been observed [3–5]. Hence, the use of antimicrobially active substances may have damaging effects on healthy cutaneous cells and impair their proliferation. A more biocompatible alternative may be packaging of antimicrobials into cyclodextrins (CDs). Cyclodextrins are cyclic oligosaccharides from the degradation of starch. Due to their ring-shaped nature they can form inclusion complexes with a big number of guest molecules [6–10]. Cyclodextrins have been successfully applied for functionalization of textiles and implant material [11–14]. For antiseptics such as chlorhexidine diacetate (CHX), iodine (IOD) and polihexanide (PHMB), the packaging into cyclodextrins could achieve a better skin compatibility, higher antimicrobial activity, and increased storage stability [15–17]. In vitro test systems provide valuable tools in the study of cytotoxic effects as well as antimicrobial activity. They use highly defined culture conditions and avoid the complex homeostatic mechanisms which occur in vivo [5], and thus allow the direct measurement of cytotoxicity, effects on bacterial growth, or the determination of cell/material interactions. However, the majority of studies on antimicrobials either

refer to antimicrobial or to cytotoxic activity, which impedes the interpretation of the results for daily practice. There are few studies that have evaluated in parallel both antibacterial power and cytotoxicity [3, 5, 18–20].

In the present study, a comprehensive in vitro approach to enable the assessment of cytotoxicity of CD-antiseptics complexes in solution is suggested. The determination of cytotoxicity is part of the initial evaluation process of materials and substances stipulated in ISO standards [21]. HaCaT cells, of a human keratinocyte cell line, have been used as a model system for proliferating keratinocytes in cytotoxicity assays. In this study, methods for the separate determination of cytotoxicity, necrosis, and apoptosis are compared to the multiplex assay ApoTox-GloTM, a screening method for determination of these three parameters in one test setup. Moreover, the antibacterial activity of cotton functionalized with CD-antiseptics complexes was determined according JIS L 1902 to evaluate their properties for supporting optimal conditions for wound healing. *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae*, and *Candida albicans* were used as model organisms to investigate the antibacterial effectiveness.

2 Materials and methods

2.1 Materials

The following antiseptics were used as solid complexes with β-cyclodextrin (β-CD, Wacker, Burghausen): chlorhexidine diacetate (CHX), iodine (IOD), and polihexanide (polyhexamethylene biguanide, PHMB) (all antiseptics: Fagron, Barsbüttel, Germany). The β-cyclodextrin-antiseptic-complexes were provided and coupled to cotton textiles by Deutsches Textilforschungszentrum Nord-West e.V. Krefeld, Germany. The solid complexes with β-cyclodextrin were prepared by the addition of the antiseptics to a hot saturated solution of β-CD. After slow cooling to room temperature the solid complexes precipitate. They were removed from the solution and dried for several hours at 40 °C [22]. To obtain the iodine complex a different procedure was used. To a solution of 19.3 mmol β-CD in 150 ml water (pH 5) a solution of 58 mmol iodine and 580 mmol KI in 50 ml water was added slowly and stirred for additional 3 h. Afterwards the solution was stored for 12 h at 0 °C. The formed brown precipitate was removed and washed with 100 ml of a solution of KI (1.2 mmol) [23]. The molar ratio of β-CD to iodine was estimated by titration with sodium thiosulfate to be 1 : 0.93.

Stock solutions of the complexes were prepared in deionised water. All solutions were sterilized by filtration (0.2 µm filter, Sartorius, Stedim Biotech, Germany).

As textile material a cotton fabric (ISO 105-F02, Testex, Zürich) was used. A β-CD derivative with a monochlorotriazinyl group (Beta W7 MCT, Cavasol W7 MCT, Wacker, Burghausen) as a reactive anchor forms stable covalent bonds with hydroxyl groups of cotton [24]. The amount of fixed β-CD on the fabric surface was in the order of 2 up to 3 % [25]. The modified cotton fabrics were immersed into solutions of the antiseptics (1 %) for 10 min. Afterwards the textiles were shortly washed with small amounts of water and afterwards ethanol. The fabrics were dried at room temperature.

Staphylococcus aureus ATCC 6538, *Escherichia coli* ATCC 35218, *Staphylococcus epidermidis* DSM 1798, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 4352 and *Candida albicans* ATCC 10231 were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). For cultivation of bacteria, special peptone and "lab-lemco" powder for preparation of caso-bouillon were obtained from Oxoid (UK). Columbia agar plates with 5 % sheep blood were purchased from Biomeriéux (France). Yeasts were cultivated in Sabouraud glucose bouillon or Sabouraud dextrose agar plates obtained from Biomeriéux (France).

The human HaCaT keratinocytes were a gift of Prof. Dr. N.E. Fusenig (DKFZ, Germany). PBS (phosphate buffered saline), DMEM (Dulbecco's modified Eagle medium), FCS (fetal calf serum), antibiotic-antimycotic mix (PSF) and Trypsin-EDTA for cell culture were obtained from PromoCell (Germany).

Annexin V-FITC, 7-amino-actinomycin D (7-AAD) staining solution, annexin V binding buffer and FITC Active Caspase-3 Apoptosis Kit were obtained from BD Biosciences (Germany). Actinomycin D was purchased from Sigma (Germany). The ApoTox-Glo^{TM} Triplex Assay was obtained from Promega GmbH (Germany).

2.2 Determination of antimicrobial activity

The determination of antimicrobial activity was performed according to the internationally recognized Japanese industrial standard (JIS L 1902: 2002, "Testing method for antibacterial activity of textiles") as described previously [3]. In brief, appropriate culture medium was inoculated with the test microbes and cultivated for 24 h at 37 °C under aerobic conditions. For experiments, 400 mg samples of the CD-antiseptic-complexes functionalized cotton were incubated with 200 µL of each test microbe suspension for 24 h at 37 °C under aerobic conditions. Samples of polyester material were used as microbial growth control because this material is known to not inhibit microbial growth. Additionally, untreated cotton and cotton equipped with β-cyclodextrin alone without antiseptics were analyzed as controls to β-CD-antiseptic-functionalized cotton samples.

Subsequently, the incubated samples were extracted in 10 mL 0.9 % NaCl solution with Tween 20. Serial dilutions were plated on agar plates, incubated for 24 h at 37 °C and colonies counted afterwards. The cfu/mL (colony forming units per milliliter) and the total microbial count of the samples (in cfu) were calculated. The growth reduction compared to the starting value was determined according to the Japanese industrial standard.

$$\text{growth reduction [log cfu]} = \log(\text{24h mean [cfu] growth control}) - \log(\text{24h mean [cfu] sample})$$

rating: no antimicrobial activity = <0.5 log microbial growth reduction
slight antimicrobial activity = 0.5–1 log microbial growth reduction
significant antimicrobial activity = >1–≤3 log microbial growth reduction
strong antimicrobial activity = >3 log microbial growth reduction

2.3 Determination of cytotoxic effects on HaCaT keratinocytes

Human HaCaT keratinocytes were cultured in DMEM supplemented with 10 % fetal calf serum and 1 % antibiotic-antimycotic mix for 7 days in 75-cm^2 cell culture flasks (Greiner, Germany) at 37 °C in a humidified atmosphere containing 5 % CO_2 atmosphere. For experiments, the cells were harvested through trypsin-EDTA treatment, seeded into 96-well microtiter plates (Greiner, Germany) at a density of 40,000 cells/cm^2. After 48 h, the culture medium was replaced by either fresh DMEM (negative control) or dilutions of CD-antiseptic-complexes. Triton X-100 (1 %) was used as positive control for cytotoxic effects. After 24 h of incubation, the determination of cell proliferation was carried out using the luminometric ATPLite Kit (Perkin Elmer Life Sciences). This assay is based on the detection of light generated by firefly luciferase. In an ATP-dependent reaction the enzyme converts D-Luciferin to Oxyluciferin. For the measurement of cellular ATP the cells were lysed with the mammalian cell-lysis buffer provided by the ATPLite Kit; the released ATP than reacts with the luciferin-luciferase system. The amount of emitted light is directly proportional to the ATP-concentration and was measured with a luminometer (LUMIstar Galaxy, BMG Labtech GmbH, Germany). Using an ATP standard curve, the ATP content [nM] of the cells can be calculated [26]. For experiments, cellular proliferation under test conditions was expressed as percent of the negative control.

To determine cytotoxic effects of the substances tested, the activity of lactate dehydrogenase (LDH) was measured in the cell culture supernatant using the cytotoxicity detection kit (Roche). LDH is released after cell damage and is a grade for necrotic cell death. The assay was run as recommended in the instructions of the manufacturer. Optical density was measured at 490 nm using a plate photometer (POLARstar Galaxy, BMG Labtech GmbH, Germany). LDH release under test conditions was expressed as percent of the positive control (HaCaT keratinocytes treated with Triton X-100).

2.4 Assessment of apoptotic effects on HaCaT keratinocytes

For determination of apoptosis by flow cytometry, 7 days old HaCaT keratinocytes, cultured as described before, were harvested through trypsin-EDTA treatment and seeded in 25-cm^2 cell culture flasks (Greiner, Germany) at a density of 20,000 cells/cm^2. After 72 h, the culture medium was replaced by either fresh DMEM (negative control) or dilutions of CD-antiseptic-complexes. To observe possible apoptotic effects, compound concentrations in the range of the individual LC_{50}-values were chosen. Actinomycin D at a concentration of 10 µg/mL served as positive control for apoptotic effects. After 24 h of incubation, cells were washed with cold PBS and harvested through trypsin-EDTA treatment. The trypsinized cells were combined with the nonadherent cells, centrifuged at 4 °C for 7 min at 1000 rpm and resuspended in cold PBS before counting using Casy-1 (Schaerfe System, Germany).

2.4.1 Annexin V-FITC/7-AAD staining

Cells were resuspended after counting in Annexin V binding buffer at a concentration of 1×10^6 cells/mL. 100 µL of the cell suspension was incubated with 5 µL Annexin V-FITC and 5 µL 7-AAD staining solution for 15 min at RT in the dark. After incubation, 400 µL of annexin V binding buffer were added and samples were analyzed within one hour on a FACSCanto flow cytometer (BD Biosciences). Data on 10,000 cells were acquired in the FITC and PerCP-Cy5.5 channels and processed using FACSDiva-software (BD Biosciences).

2.4.2 Active caspase-3 staining

1×10^6 cells were washed twice with cold PBS and fixed with 500 µL Cytofix/Cytoperm (BD Bioscience) for 20 min on ice in the dark. After washing twice with 500 µL perm/wash buffer (BD Bioscience), cells were stained with the FITC anti-active caspase-3 monoclonal antibody (BD Bioscience) for 30 min at RT in the dark. After staining, cells were washed once and resuspended in perm/wash buffer. Fluorescence data on 10,000 cells were acquired in

the FITC channel and processed using FACSDiva-software (BD).

2.5 Multiplex analysis of cell viability and death

The ApoTox-Glo™ Triplex Assay (Promega GmbH, Germany) was used to measure viability, cytotoxicity and apoptosis of HaCaT keratinocytes after 24 h of incubation by a multiplex assay [27]. The assay was run as recommended in the instructions of the manufacturer.

Viability of cells is determined by protease activity, which is restricted to intact, viable cells [28]. The cell-permeant fluorogenic peptide substrate Gly-Phe-aminofluorocoumarin (GF-AFC) is cleaved proteolytically to AFC in intact cells, where it generates a fluorescent signal. Cell viability was measured at 400 nm_{ex}./500 nm_{em}. with a plate fluorometer (FLUOstar Galaxy, BMG Labtech GmbH, Germany).

Cytotoxicity is measured by protease activity of a death cell protease that is released from nonviable cells after loss of membrane integrity [28]. The fluorogenic peptide substrate bis-Ala-Ala-Phe-rhodamine 110 (bis-AAF-R110), which does not permeate intact cell membranes, is cleaved by the death cell protease to the fluorescent product R110 that was measured at 485 nm_{ex}/520 nm_{em}. with a plate fluorometer (FLUOstar Galaxy, BMG Labtech GmbH, Germany).

Apoptosis-related caspase-3/7 activity was determined in a luminometric assay. Following the cleavage of the caspase-3/7-specific substrate benzyloxycarbonyl-Asp-Glu-Val-Asp-aminoluciferin, luciferase can catalyze the reaction of released aminoluciferin, which generates a luminescence signal that was measured with a plate luminometer (LUMIstar Galaxy, BMG Labtech GmbH, Germany).

2.6 Statistics

Studies were performed in duplicate, and each sample was measured in three (flow cytometric testing and antimicrobial testing) or four (ATP and LDH testing) replicates, respectively. The ApoTox-Glo™ Triplex Assay was performed in quadruplicate with measuring four replicates. All data are given as mean ± SD. LC_{50} values were calculated using Origin® 7.5 (OriginLab, Northampton, U.S). One-way analysis of variance was carried out to determine statistical significances (Microsoft® Excel 2000). Differences were considered statistically significant at a level of $P < 0.05$. Asterisks indicate significant deviations from the control ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

3 Results

3.1 Antimicrobial activity

All β-CD-antiseptic-functionalized cotton samples exhibited distinct antimicrobial effects against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* (Table 1). Functionalization with β-CD-PHMB proved most effective as samples demonstrated a strong antimicrobial activity against all test species (log-reduction > 3). β-CD-CHX was able to inhibit or reduce the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*,

Table 1 Determination of the antimicrobial activity of functionalized cotton samples according to JIS L 1902

Cotton		*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*E. coli*	*K. pneumoniae*	*C. albicans*
Untreated	Microbial growth [%]	98.7 ± 1.8	102.1 ± 1.7	105.7 ± 1.7	98.3 ± 0.7	104.2 ± 1.2	97.3 ± 2.0
	Growth reduction [log cfu]	0.09	0	0	0.14	0	0.18
	Activity	None	None	None	None	None	None
+β-CD	Microbial growth [%]	85.8 ± 2.0	99.1 ± 0.6	103.0 ± 1.3	104.1 ± 1.0	104.2 ± 0.4	102.9 ± 1.6
	Growth reduction [log cfu]	0.99	0.06	0	0	0	0
	Activity	Slight	None	None	None	None	None
+β-CD-PHMB	Microbial growth [%]	0	0	18.8 ± 13.6	0	0	0
	Growth reduction [log cfu]	6.96	6.78	6.64	8.07	7.64	6.50
	Activity	Strong	Strong	Strong	Strong	Strong	Strong
+β-CD-CHX	Microbial growth [%]	0	12.8 ± 18.1	99.7 ± 0.4	60.4 ± 3.0	0	63.5 ± 2.6
	Growth reduction [log cfu]	6.96	5.91	0.02	3.20	7.64	2.37
	Activity	Strong	Strong	None	Strong	Strong	Significant
+β-CD-IOD	Microbial growth [%]	49.6 ± 9.4	66.8 ± 3.0	74.3 ± 31.2	68.2 ± 18.7	79.8 ± 38.3	96.7 ± 1.6
	Growth reduction [log cfu]	3.51	2.25	2.10	2.57	1.55	0.22
	Activity	Strong	Significant	Significant	Significant	Significant	None

Klebsiella pneumoniae, and *Escherichia coli* (log-reduction > 3) and was further effective against the yeast *Candida albicans* (log-reduction = 2.37) while it failed to affect the growth of *Pseudomonas aeruginosa*. In contrast, β-CD-IOD exhibited a strong antibacterial effect against *Staphylococcus aureus* (log-reduction > 3) and was able to significantly reduce the growth of *Staphylococcus epidermidis* (log-reduction = 2.25), *Klebsiella pneumoniae* (log-reduction = 1.55), *Escherichia coli* (log-reduction = 2.57), and *Pseudomonas aeruginosa* (log-reduction = 2.1). For β-CD-IOD no antifungal effect was observed. Untreated cotton and cotton equipped with β-CD alone did not significantly affect microbial growth.

3.2 Determination of cytotoxicity by ATP measurement

Cell viability and proliferation of HaCaT keratinocytes incubated for 24 h with increasing concentrations of β-CD and β-CD-antiseptic-complexes were determined by detection of the cellular ATP content. As shown in Fig. 1, a dose-dependent decline of cell viability is observed. LC_{50} concentrations (half minimum lethal concentrations after 24 h) were calculated for each compound tested (Table 2). For β-CD and β-CD-IOD, there was a distinct decrease of cell viability at concentrations of ≥5 mg/mL. For β-CD-CHX, cell viability of HaCaT keratinocytes decreased significantly after incubation for 24 h with concentrations of ≥2 mg/mL. β-CD-PHMB was the most cytotoxic compound of the tested β-CD-antiseptic-complexes. Here, a decrease of HaCaT keratinocyte viability was visible after incubation with concentrations of ≥0.5 mg/mL for 24 h. The LC_{50} value of β-CD-IOD (6.28 mg/mL) was comparable to β-CD (6.70 mg/mL) whereas LC_{50} values of β-CD-CHX and β-CD-PHMB were lower (2.53 and 0.33 mg/mL, respectively).

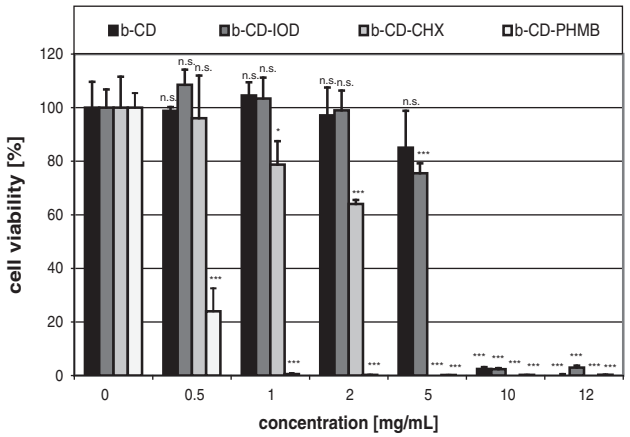

Fig. 1 Determination of cell viability of β-CD and β-CD-antiseptic-complexes on HaCaT keratinocytes after 24 h incubation by analysis of the cellular ATP content. With β-CD and β-CD-IOD the viability of the HaCaT keratinocytes after 24 h incubation is significantly reduced at concentrations of > 5 mg/mL, whereas with β-CD-CHX and β-CD-PHMB the viability is reduced at concentrations of ≥2 mg/mL and ≥ 0.5 mg/mL, respectively. Differences were considered statistically significant at a level of $P < 0.05$. Asterisks indicate significant deviations from the medium control (*$P < 0.05$; **$P < 0.01$; *** $P < 0.001$)

Table 2 Comparison of LC_{50} value of HaCaT keratinocytes after 24 h incubation with β-CD-antiseptic-complexes calculated by ATP determination and ApoTox-Glo^{TM} triplex assay

Compound	LC_{50} after 24 h incubation in mg/mL		
	Determination of ATP	Triplex	*P*
β-CD	6.70 ± 0.33	6.92 ± 1.08	0.685
β-CD-CHX	2.53 ± 0.07	3.85 ± 1.36	0.105
β-CD-IOD	6.28 ± 0.52	6.70 ± 1.06	0.934
β-CD-PHMB	0.33 ± 0.04	0.32 ± 0.09	0.789

One-way analysis of variance resulted in no significant statistical differences between the results of both methods ($P > 0.05$)

3.3 Determination of necrosis by LDH measurement

Additionally, the LDH leakage into the cell culture supernatant was determined. Figure 2 shows increasing LDH values at higher substance concentrations and good correlations to the dose-dependent decreasing cellular ATP contents. The highest LDH release of HaCaT keratinocytes at low substance concentrations of ≤1 mg/mL was measured for β-CD-PHMB. With β-CD-CHX, there was an increase in LDH release after incubation with concentrations of >2 mg/mL for 24 h resulting in LDH levels comparable to the positive control Triton-X. β-CD-IOD is the least cytotoxic β-CD-antiseptic-complex tested with a LDH release comparable to β-CD. Here, an increase of LDH release is observed at concentrations of >5 mg/mL (Fig. 2).

3.4 Determination of apoptosis by flow cytometry

For exploring the mode of cell death, two independent flow cytometric methods for detection of apoptosis were applied. To observe possible apoptotic effects, compound concentrations in the range of the individual LC_{50}-values were chosen. In Fig. 3 and Table 3 the flow cytometric results of AnnexinV/7-AAD staining after 24 h incubation of HaCaT keratinocytes with the substances tested is shown. In the medium control >90 % of the HaCaT population were healthy cells with AnnexinV$^{(-)}$/7-AAD$^{(-)}$ staining. A population of <5 % were apoptotic cells with AnnexinV$^{(+)}$/7-AAD$^{(-)}$ staining and <5 % dead cells with AnnexinV$^{(+)}$/7-AAD$^{(+)}$ staining. Incubation of HaCaT cells with 10 µg/mL ActinomycinD for 24 h resulted in a noticeable apoptotic population of 46.6 % of the cells with AnnexinV$^{(+)}$/7-AAD$^{(-)}$ staining and 30.1 % dead cells with AnnexinV$^{(+)}$/7-AAD$^{(+)}$ staining.

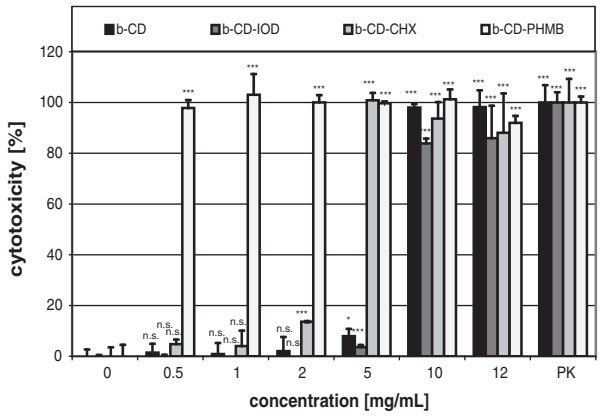

Fig. 2 Determination of cytotoxicity of β-CD and β-CD-antiseptic-complexes on HaCaT keratinocytes after 24 h incubation by analysis of LDH release. The highest LDH release at low substance concentrations of ≥ 0.5 mg/mL was observed for β-CD-PHMB. For β-CD-CHX the LDH release increased at concentrations of ≥ 2 mg/mL. The results of β-CD-IOD and β-CD were comparable with high LDH release at concentrations of > 5 mg/mL. Differences were considered statistically significant at a level of $P < 0.05$. Asterisks indicate significant deviations from the medium control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

Incubation with β-CD and β-CD-IOD at different concentrations for 24 h lead to distinct apoptotic cell populations. As seen in Fig. 3 incubation with 10 mg/mL β-CD resulted in 55.5 % apoptotic cells and incubation with 7.5 mg/mL β-CD-IOD in 36.6 % apoptotic cells. After incubation with β-CD-CHX and β-CD-PHMB at different concentrations no enhanced apoptosis was detected with a cell distribution comparable to the medium control.

To verify these results, a second, independent method for detection of apoptosis was applied. HaCaT keratinocytes were incubated with similar concentrations to the previous experiment and flow cytometric detection was done by intracellular active caspase-3 staining. As shown in Fig. 4, the results of the AnnexinV/7-AAD method were confirmed. No enhanced apoptosis of HaCaT keratinocytes was detected after incubation with β-CD-CHX and β-CD-PHMB at concentrations of 6.0 mg/mL and 0.5 mg/mL, respectively, with values comparable to the medium control (Table 3). In contrast, after incubation with 10 mg/mL β-CD for 24 h 85.6 % of the cells were active-caspase3-positiv, after incubation with 7.5 mg/mL β-CD-IOD even 99.7 % of the keratinocytes were apoptotic. Similar results were

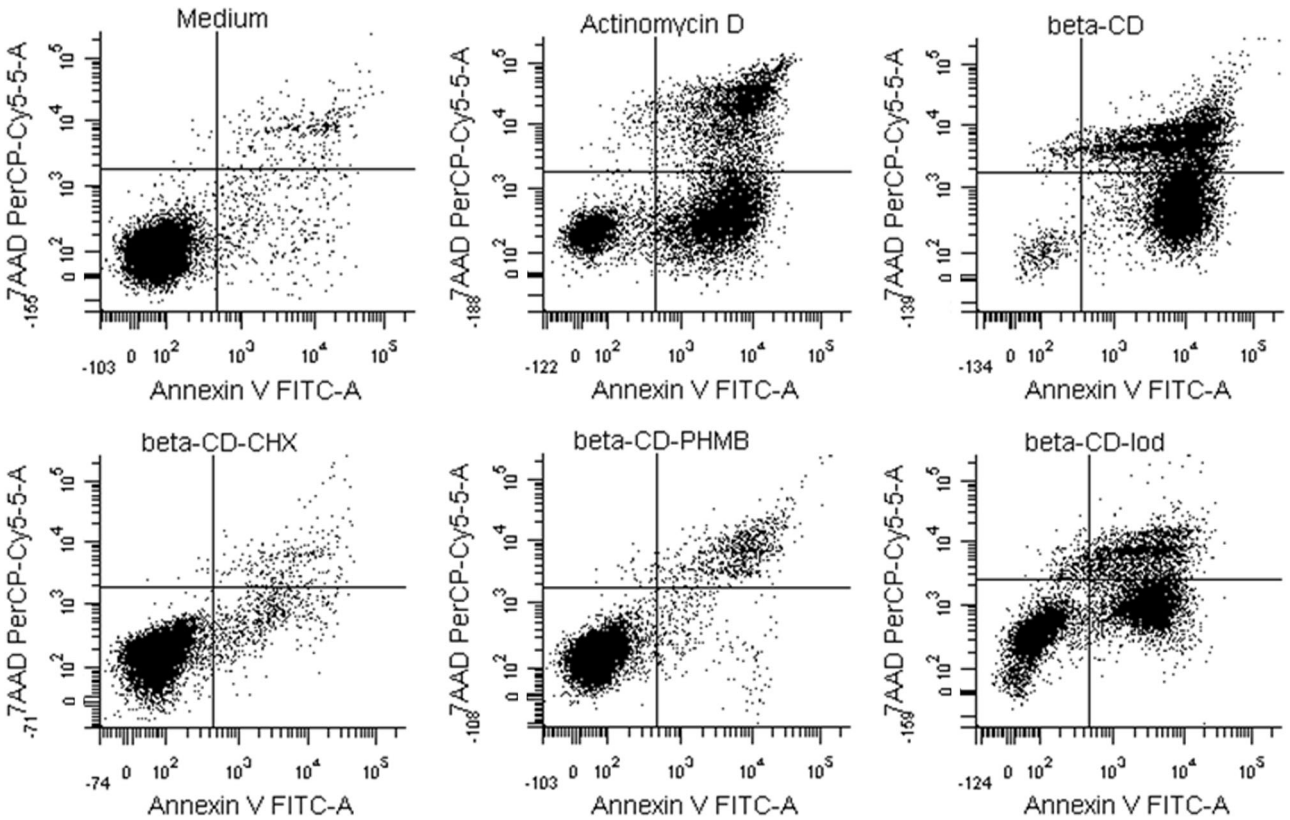

Fig. 3 Exemplary presentation of flow cytometric AnnexinV/7-AAD staining after 24 h incubation of HaCaT keratinocytes with Medium, ActinomycinD (10 µg/mL), β-CD (10 mg/mL), β-CD-CHX (6.0 mg/mL), β-CD-PHMB (0.5 mg/mL) and β-CD-IOD (7.5 mg/mL). Compound concentrations in the range of the individual LC_{50}-values were chosen. For β-CD-CHX and β-CD-PHMB no enhanced apoptosis was determined with a cell distribution comparable to the medium control. For β-CD and β-CD-IOD enhanced apoptosis was detected with a cell distribution similar to the positive control ActinomycinD

Table 3 Flow cytometric results of determination of apoptosis in HaCaT keratinocytes after 24 h incubation with β-CD-antiseptic-complexes

Substance	Concentration	Annexin V/ 7-AAD			Active caspase-3	
		Live %	Apoptotic	Dead	Negative %	Positive
Medium		93.2	3.9	2.8	98.1	1.0
Actinomycin D	10 µg/mL	22.4	46.6	30.1	29.8	69.9
β-CD	10 mg/mL	2.7	55.5	40.6	12.0	85.6
β-CD-CHX	6.0 mg/mL	92.1	5.4	2.4	97.0	2.5
β-CD-PHMB	0.5 mg/mL	88.4	2.7	8.6	98.8	1.2
β-CD-IOD	7.5 mg/mL	42.7	36.6	18.9	0.2	99.7

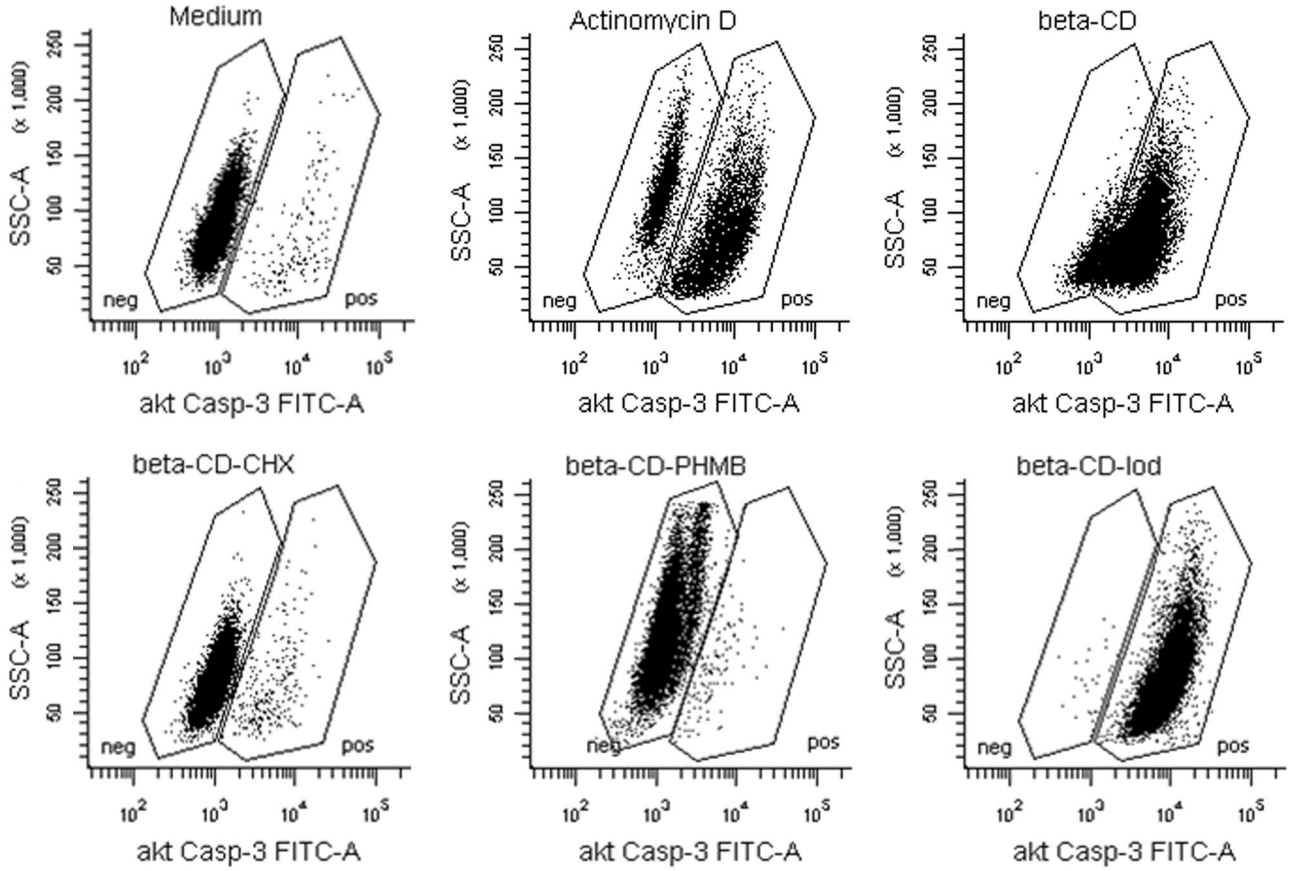

Fig. 4 Exemplary presentation of flow cytometric active-caspase-3 staining after 24 h incubation of HaCaT keratinocytes with Medium, ActinomycinD (10 µg/mL), β-CD (10 mg/mL), β-CD-CHX (6.0 mg/mL), β-CD-PHMB (0.5 mg/mL) and β-CD-IOD (7.5 mg/mL). Compound concentrations in the range of the individual LC_{50}-values were chosen. For β-CD-CHX and β-CD-PHMB no enhanced apoptosis was determined with a cell distribution comparable to the medium control. For β-CD and β-CD-IOD enhanced apoptosis was detected with a cell distribution similar to the positive control ActinomycinD

achieved with the positive control ActinomycinD which resulted in 69.9 % active-caspase3-positive cells.

3.5 Determination of viability, cytotoxicity, and apoptosis by the ApoTox-Glo™ triplex assay

In Fig. 5 and Table 2 the results of the ApoTox-Glo™ Triplex Assay of HaCaT keratinocytes after 24 h incubation with increasing concentrations of β-CD and β-CD-antiseptic-complexes are shown. LC_{50} values, calculated from the viability curve of the assay, were comparable for β-CD-IOD (6.70 mg/mL) and β-CD (6.92 mg/mL). Incubation with β-CD-CHX and β-CD-PHMB induced more cytotoxic effects on HaCaT keratinocytes with LC_{50} values of 3.85 and 0.32 mg/mL, respectively. Apoptotic cell death was detected after incubation of HaCaT keratinocytes with β-CD

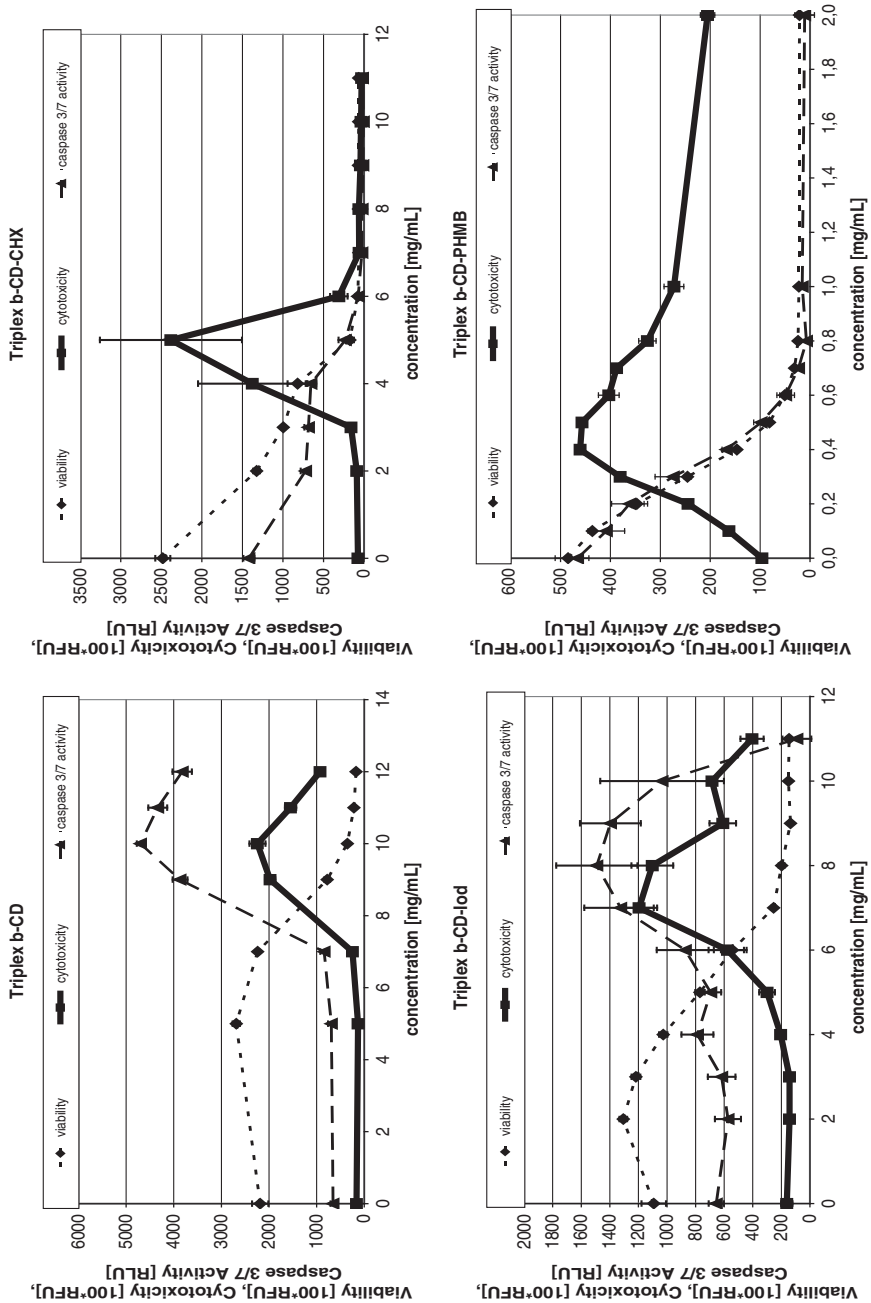

Fig. 5 ApoTox-Glo^{TM} Triplex Assay of β-CD-antiseptic-complexes on HaCaT keratinocytes after 24 h incubation. LC_{50}-values, calculated from the viability curve of the assay, were comparable for β-CD and β-CD-IOD (6.92 and 6.70 mg/ml, respectively). Incubation with β–CD-CHX and β-CD-PHMB induced more cytotoxic effects on HaCaT keratinocytes with LC_{50}-values of 3.85 and 0.32 mg/mL, respectively. Apoptotic cell death was detected after incubation of HaCaT keratinocytes with β-CD and β-CD-IOD

and β-CD-IOD. At concentrations higher than the LC_{50} values of the compounds an increase in caspase 3/7-activity with a peak value at 10 mg/mL and 8 mg/mL, respectively followed by a falling curve with increasing incubation concentrations was visible. No apoptosis was detected after incubation of HaCaT keratinocytes with β-CD-CHX and β-CD-PHMB.

Cytotoxicity, measured by the activity of a cell death protease, showed a reverse curve compared to the viability curve with increasing concentrations up to the LC_{50} values of the compounds. However, at higher concentrations a sudden decline of the activity of the cell death protease could be detected. This effect is most obvious after incubation of HaCaT keratinocytes with increasing concentrations of β-CD-CHX (Fig. 5).

4 Discussion

The use of antimicrobially active compounds is a promising approach in health care settings, especially with respect to an increasing number of bacterial strains such as MRSA that are resistant to antibiotics. Functionalized textiles can be used in wound management to reduce the microbial burden in the wound area, to prevent wound infections, and to avoid cross-contamination between patients.

In this study, the antimicrobial effect of cotton functionalized with β-CD-antiseptic-complexes was tested according to the Japanese industrial standard as described previously [3]. The guest molecules in the cyclodextrin complexes were the antiseptics chlorhexidine diacetate (CHX), iodine (IOD), and polihexanide (PHMB). The selected microorganisms are typical for the microflora in chronic wounds and nosocomial infections [29, 30]. For all β-CD-antiseptic functionalized cotton samples a strong to significant antibacterial effect on the Gram-positive bacteria *S.aureus* and *S.epidermidis* as well as on the Gram-negative bacteria *K.pneumoniae* and *E.coli* was proven. β-CD-CHX and β-CD-PHMB were further effective against the yeast *C. albicans*. The growth of *P.aeruginosa* could be reduced significantly to strongly by β-CD-IOD and β-CD-PHMB. The antimicrobial effects can be fully attributed to the antiseptics because untreated cotton and cotton equipped with β-CD did not significantly affect microbial growth. These results are in concordance with Finger et al. [31, 32] who studied the antimicrobial properties of different cyclodextrin-antiseptic-complexes in solution.

One important prerequisite for application of these highly antimicrobially active functionalized textiles in wound healing is the proof of biocompatibility of the compounds to avoid damaging effects on healthy cutaneous cells during application. In this study, HaCaT cells were used as a model system for proliferating keratinocytes in different cytotoxicity assays. The viability of the HaCaT keratinocytes after 24 h incubation with β-CD-antiseptic-complexes in solution was measured by a luminometric ATP assay. Additionally, cytotoxicity of β-CD-antiseptic-complexes after incubation with HaCaT keratinocytes was measured by LDH release. The mode of cell death was proven by two independent flow cytometric methods for detection of apoptosis. Although all applied methods are based on different analytical techniques, consistent results were obtained.

For β-CD and β-CD-IOD, the induction of apoptosis as the mode of cell death of HaCaT keratinocytes after incubation with these complexes could be proven by two independent flow cytometric methods. In healthy cells, there is an asymmetric distribution of plasma membrane phospholipids between the inner and outer layer. During apoptosis, there is a flip of phosphatidylserine from the inner to the outside leaflet of the membrane. This exposition of phosphatidylserine can be detected by binding to the anticoagulant protein AnnexinV [33]. A central role in apoptosis plays the activation of the caspase cascade. Both, the extrinsic and intrinsic apoptosis pathways lead to the activation of effector caspases such as caspase-3, -6, and -9 [34]. The activated form of caspase-3 could be detected in this study by intracellular staining with a specific antibody as a marker for apoptosis. These results are in concordance with Schonfelder et al. [35] who described the apoptotic death of keratinocytes after incubation with 10 mg/mL β-CD for 24 h. Different authors observed the induction of apoptosis by iodine on human epidermal fibroblasts [36], breast cancer cells [37] and thyroid cells [38, 39]. Hence, the effects of β-CD-IOD observed in this study are probably a combination of the effects of β-CD and iodine. The complexes β-CD-CHX and β-CD-PHMB did not induce apoptosis in HaCaT keratinocytes, therefore toxic effects on HaCaT keratinocytes are probably induced by necrosis. After incubation for 24 h with 6 mg/mL β-CD-CHX and 0.5 mg/mL β-CD-PHMB, respectively the rate of AnnexinV binding and active caspase-3 staining was comparable to the medium control.

Cytotoxic effects of chlorhexidine on fibroblasts [40, 41] and osteoblasts and endothelial cells [41] have been previously described. Interestingly, there is a dose-dependent mode of cell death, leading to apoptosis of the cells when using low concentrations of chlorhexidine and to necrosis if higher chlorhexidine concentrations are applied. The cytotoxic effects of chlorhexidine are caused by alteration of the mitochondrial membrane potential and the actin cytoskeletal assembly, by triggering of intracellular Ca^{2+} increase, the generation of reactive oxygen species (ROS) and enhanced stress of the endoplasmic reticulum [40, 41].

It is still a matter of research by which mechanism PHMB kills mammalian cells. Rohner et al. [42] reported a

significant increase in LDH release to the culture medium after short incubation time of PHMB on human chondrocytes and swelling of the cells. Creppy et al. [43] investigated the influence of PHMB on Caco-2 cells, Neuro-2A cells and hepatocellular carcinoma cells. At very low PHMB concentrations they observed a hormesis-like effect with about 20 % increase in cell proliferation, an effect confirmed by Wiegand et al. [44]. At cytotoxic concentrations the cells burst followed by rapid LDH release. There was no increase in DNA fragmentation, oxidative stress and no activation of caspase-3, so the authors conclude that cells are not undergoing apoptosis but cell death occurs by necrosis as a result of membrane lysis and leakage of enzymes and cytokines.

In parallel to the independent viability, cytotoxicity, and apoptosis assays the multiplex assay ApoTox-Glo^{TM} was tested for comparing the information obtained by these different methods. In the ApoTox-Glo^{TM} assay viability, cytotoxicity, and apoptosis are determined in one 96-well plate by fluorometric measurement of the activities of a life-cell protease and a death cell protease followed by the luminometric detection of caspase-3/7 activity. The main benefit of this multiplex assay compared to the independent assays is the time and cost saving format of the test because all three assays are done in one plate with the same cells. This is advantageous for high-throughput-screening (HTS). However, a disadvantage of this assay is the finite enzymatic half-life of the dead cell protease that can limit their utility especially during long term incubations. The enzymatic decay can lead to underestimation of the absolute level of cytotoxicity [45].

Using the viability curve of the ApoTox-Glo^{TM} assay LC_{50} values comparable to the LC_{50} values obtained from the independent ATP-viability assay were calculated (Table 2). For detection of cytotoxicity the activity of a death cell protease released from nonviable cells is measured in this assay. With increasing compound concentrations there is an increase in this protease activity but at values higher than the LC_{50} values a decline of the death cell protease activity was observed. This effect is most noticeable during incubation of HaCaT keratinocytes with β-CD-CHX. In contrast, in the independent cytotoxicity assay the LDH values at cytotoxic concentrations are stable over at least 48 h and comparable to the positive control Triton X-100. This confirms that the death cell protease measured in the ApoTox-Glo^{TM} assay is not stable, so at higher compound concentrations which are cytotoxic already after 1 h incubation no cell death protease activity can be detected after 24 h incubation. For measuring cytotoxicity the ApoTox-Glo^{TM} assay is suitable only for screening but for quantitative calculation of cytotoxicity the more stable LDH assay in combination with a positive control is advisable.

When measuring the apoptosis-related caspase-3/7 activity with the ApoTox-Glo^{TM} assay apoptosis was detected during incubation of the HaCaT keratinocytes with β-CD and β-CD-IOD at concentrations comparable to the values determined by the flow cytometric methods. As observed for the cell death protease activity, there is a decrease of the caspase-3/7 activity with increasing compound concentrations after a peak during apoptosis. For compounds such as β-CD-CHX and β-CD-PHMB with necrotic effects on the cells there is a constant decrease of the caspase-3/7 activity with increasing compound concentrations. Presumably, this results from decreasing cell numbers at cytotoxic concentrations. Displaying the ApoTox-Glo^{TM} assay data in a single diagram as proposed by the manufacturer and Niles et al. [28] has the disadvantage of combining different absolute fluorescence and luminescence values in one graph, but relative quantitative viability and cytotoxicity data as obtained from the independent ATP and LDH assays cannot be generated. So the ApoTox-Glo^{TM} assay is as mentioned before a good tool for screening unknown compounds for biocompatibility but for quantitative data more detailed methods as the ATP and LDH assays and flow cytometric methods are advisable.

The prerequisite for application of cyclodextrin-functionalized antimicrobial textiles in wound healing is the combination of high antimicrobial activity and low cytotoxicity on dermal cells. Due to stable covalent binding of β-CD to the cotton carrier the host of the complex is washfast fixed to the textile [24] and a release of β-CD in to the systemic circulation is unlikely. The antiseptics used in this study form reversible inclusion complexes with β-CD. It is possible that the antimicrobials fixed in the complex are released from the functionalized textile to a certain amount during usage and washing. Further tests are necessary to evaluate release capabilities. In our studies, β-CD-IOD was the least cytotoxic compound tested, but compared to the other β-CD-antiseptics-complexes it was also the least antimicrobial active compound. So there was no effect of the β-CD-IOD-functionalized cotton against *C. albicans* and only significant effects against *S. epidermidis*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae*. Additionally, apoptotic effects were detected during incubation of HaCaT keratinocytes with β-CD-IOD. β-CD-CHX-functionalized cotton showed no antibacterial effects against *P. aeruginosa* and only significant effects against *C. albicans*. β-CD-PHMB was the most cytotoxic solution compared to β-CD-CHX and β-CD-IOD, but the β-CD-PHMB-functionalized cotton was most effective against all the microorganisms tested. It is reasonable to conclude that there is an option to use only low concentrations of the β-CD-PHMB-complex on textiles obtaining a high antimicrobial activity and minimizing cytotoxic effects, simultaneously.

5 Conclusion

The high antimicrobial activity of β-CD-antiseptic-complex-functionalized textiles observed promises an eminent potential of these compounds for medical applications. In addition, cytotoxicity testing results for CD-antiseptics complexes in solution suggest a good cell compatibility. Nonetheless, it is recommendable to run further tests on cellular effects when these compounds are fixed to textiles to evaluate release capabilities. This can be done by measuring extracts of these textiles obtained according to DIN EN ISO 10993-12. Lastly, the comprehensive testing systems used in these studies for determination of biocompatibility are suitable for obtaining robust data on cell viability, cytotoxicity and mode of cell death. What is more, similar results were yielded using separate methods for determination of cytotoxicity, necrosis, and apoptosis when compared to the multiplex assay ApoTox-Glo™.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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