ANTIBACTERIAL PERFORMANCE OF CHLORHEXIDINE ACETATE TREATED PLAIN COTTON AND β-CYCLODEXTRIN-COTTON

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ABSTRACT
Cotton was treated with β-cyclodextrin via a crosslinker 1, 2, 3, 4, butane tetracarboxylic acid. β-cyclodextrin attached cotton and plain cotton was treated with the antimicrobial agent Chlorhexidine acetate. The difference in amount of Chlorhexidine acetate loaded onto the two types of fabrics for same application concentrations was noted. These two types of fabrics were then tested for antibacterial performance. The antibacterial activity was tested according to the JIS L 1902 standard using the ATCC 11229 *E.coli* bacteria. Antibacterial tests indicated slow release of Chlorhexidine acetate (CHXA) from β-cyclodextrins attached cotton in comparison with the rapid release of CHXA from plain cotton. This property of slow release in the former has applications wound dressings.

Key Words: Antibacterial textiles, Chlorhexidine acetate, cotton, β-cyclodextrin.

INTRODUCTION
The aim of this work was to functionalize cotton substrate with an antimicrobial agent to obtain an antimicrobial textile. This functionalization was achieved via the use of a host-guest system. The attachment of the host molecule β-cyclodextrin (BCD) onto cotton substrate was achieved via a crosslinker 1, 2, 3, 4-butane tetracarboxylic acid (BTCA) and with a catalyst sodium hypophosphite (SHPI). Chlorhexidine acetate (CHXA) was the chosen as the guest antimicrobial agent due to its molar mass of 625.55 g/mol apart from the presence of hydrophobic groups in its structure. Both of these two factors fulfil the requirements necessary for complexation of any guest molecules inside the hydrophobic BCD cavity [1, 2]. Additionally, CHXA is also known to be safe in terms of development of microbe resistance [3] and CHXA is widely used disinfectant and available commercially.

CHXA and BCD homogenous tests were first conducted to check for complexation with UV vis spectroscopy (Cary 100). These tests were compared with homogenous tests done with Chlorhexidine base (CHXB) and BCD. CHXA was then loaded onto BCD treated fabric (BCD-cotton) and plain cotton fabric to make an antimicrobial textile. Antibacterial tests were conducted for two types of functionalized fabrics viz CHXA-BCD-cotton vs. CHXA-cotton. In this paper, while referring to the a specific type of Chlorhexidine, an acronym CHXA or CHXB is used and while referring to the molecule in general the generic name Chlorhexidine is used.

2. EXPERIMENTAL

2.1.Materials

100 % plain woven, scoured cotton used for this work was purchased from WFK testgewebe, Germany. BCD was obtained as a gift from Roquette, France. BTCA, SHPI, CHXA and CHXB were obtained from Sigma Aldrich. ATCC 11229 *E. coli* bacteria was obtained from LGC standards.

2.2. Methods

2.2.1. Homogenous tests with UV vis spectroscopy

The difference between CHXA and CHXB is that CHXA is the salt form of CHXB and therefore contains an additional acetate group. The solubility of CHXB in water is 0.008 % w/v in comparison with the 1.9 % w/v of CHXA. The molar mass of CHXB is also lower at 505.45 g/mol in comparison with the 625.55 g/mol of CHXA. The aim of these experiments was to verify if any differences in complexation could be noted via UV vis spectroscopy between CHXA and BCD and between CHXB and BCD due to these differences in molar mass and water solubility between the two types of Chlorhexidine.

For these experiments, solutions of CHXA and BCD were taken. The final concentration of CHXA was always 30 µM while the concentrations of BCD varied from 30 µM to 7.5 mM. UV vis spectroscopy scans of these mixtures were performed between 200nm to 800nm covering spectrum of UV and visible range. The same was done for CHXB and BCD complexation tests. 7.5 mM was the maximum concentration in the final CHXA and BCD solutions prepared due to the limitations in BCD solubility in water.

2.2.2. CHXA loading onto BCD-cotton vs. plain cotton

Fixation of BCD on cotton was performed according to the optimum conditions gathered from literature and previous work [4]. A 25 cm by 25 cm cotton fabric was treated with a BCD concentration of 100 g/l, 30 g/l of BTCA and with the catalyst SHPI (at ratio of 1:0.3 mole ratio between BTCA and SHPI). The cotton sample was pre-dried at 110°C for 10 min and cured at 160°C for 5 min. This cotton sample was then rinsed and dried at room temperature. The dried BCD-cotton sample was then treated with CHXA (CHXA-BCD-cotton).

The treatment of CHXA on plain cotton and BCD-cotton ranged from 0.01 g/l to 2.5 g/l. The liquor to cloth ratio (LCR) for these experiments was 10:1 and the experiments were done at 40°C for 30 min in a water bath. The amount of CHXA loaded onto the two fabrics was determined by measuring the absorbances of CHXA in exhaust bath during the experiments. From the differences in concentrations of CHXA in treatment beakers from the start to the end of the experiment, the loading of CHXA (mg/g of fabric) on the fabric could be calculated.

2.2.3. Antibacterial testing

For the antibacterial tests, 0.25g of the CHXA-BCD-cotton and CHXA-cotton was taken and inoculated with 200µl of 1-3 X 10^6 CFU (Colony Forming Unit)/ml of *E.coli* bacteria. The concentration was obtained by the measurement of the optical density of inoculum with a WPACO 8000 Biowave personal cell density meter (Biochrom, UK) at 600 nm. The required optical density was obtained after diluting the bacterium grown until 10^8 CFU/ml with Luria Broth medium.

After the inoculation, the samples were placed on the inside of a petri dish lid and then covered with an inverted agar filled petri dish bottom and sealed with paraffin tape. This was done to prevent drying of the sample during the incubation. The samples were placed in an incubator operating at 37 °C for 24 hours.

After the incubation period, the samples were taken out and shaken in 20 ml of physiological saline containing 2 g/l of nonionic surfactant (Triton x100) as prescribed by the standard. 1 ml of shake out solution was then serially diluted in 9 ml of physiological saline for the required number of dilutions and then 0.1 ml was plated. The number of colonies grown on the plate was counted the next day to calculate the bacteria eluted from each specimen (through plate count method). The amount of bacteria eluted from control samples (these are only BCD-cotton and plain cotton respectively) at time 0 (CFU*0 and CFU0) was 1-3 X 10^6 CFU/ml. It was determined by eluting the bacteria immediately from the samples after inoculation by the above described procedure, however, without the incubation step. The lower limit of the plate count method was 6 x 10^3 CFU per specimen below which the CFUs obtained were considered zero. The reason for this being that plates with colonies only 30-300 were considered and plates with colonies below 30 were considered 0 CFUs. The antibacterial activity was also estimated using the bacteriostatic formula described in JIS L 1902 standard as shown below [5].

Antibacterial activity= $(\log CFU^{*}_{t} - \log CFU^{*}_{0}) - (\log CFU_{t} - \log CFU_{0})$ (1)

where CFU^{*}_{0} and CFU^{*}_{t} are the Colony Forming Units (CFU) obtained on the control sample at time 0 and time t. CFU_{0} and CFU_{t} are CFU obtained on the treated sample at time 0 and time t.

3. RESULTS AND DISCUSSIONS

3.1. Homogenous complexation study

As mentioned earlier, the differences between the two forms of Chlorhexidine (CHXA and CHXB) is related to the molar mass and solubility. Both these two types of Chlorhexidine molecules show an absorbance peak at 254nm. In the complexation experiments no change in the wavelength of this absorbance peak was noted of solutions mixed at 1:1 to 1:4 molar ratios between BCD and CHXA (not shown here). This was also the case when BCD solution concentrations were steadily increased to 0.625 mM of BCD while the concentration of CHXA remained constant at 30 µM. A shift in the wavelength of the absorbance peak was noted starting from 1.25 mM of BCD indicating the start of complexation between the CHXA and BCD molecule. This shift was of 2nm at 1.25 mM of BCD and at 4 nm at 2.5 mM of BCD. As the BCD concentration was further increased, no further horizontal shift in the wavelength was seen. This can be noted in Figure 1A. This same behavior was noted for CHXB (see Figure 1B). This data led us to concluded that though both the forms of Chlorhexidine are different in terms of solubility and molar mass, they do not seem to have a big influence on the complexation with BCD. The pattern of complexation is identical. If there had been any differences, then the shift in wavelength would have been delayed or noted earlier. We also concluded from these experiments that a molar excess of BCD was needed for complexation with CHXA and CHXB in homogenous solutions.

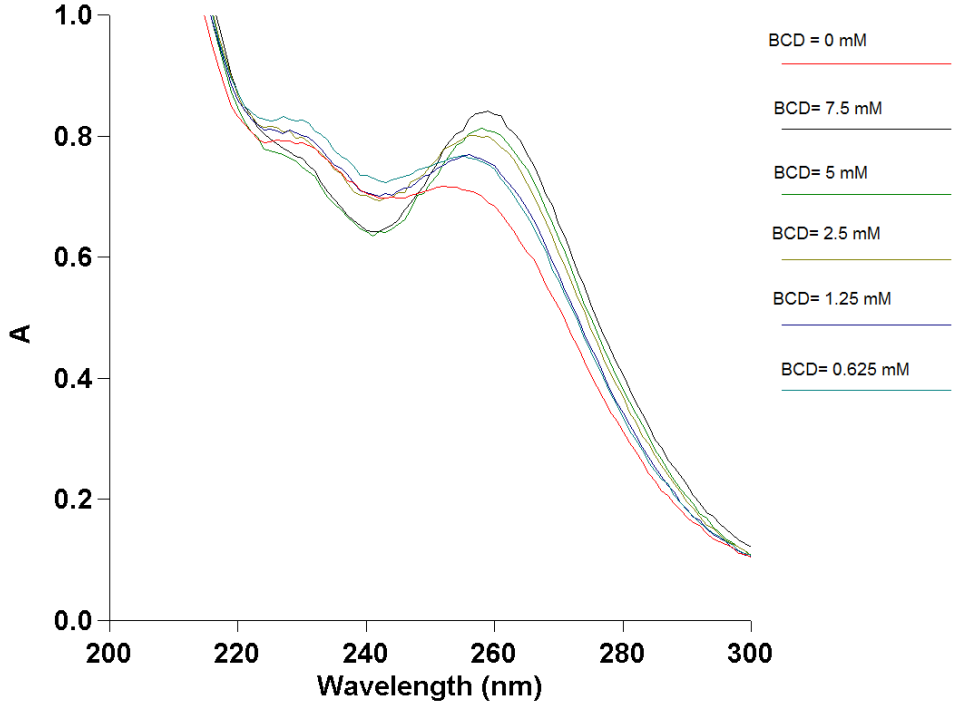

Figure 1A: The shift in the wavelength of the absorbance peak of CHXA from 254nm to 258nm when CHXA is complexed with certain concentrations of BCD. The legend describes the concentration of BCD used. A refers to the absorbance.

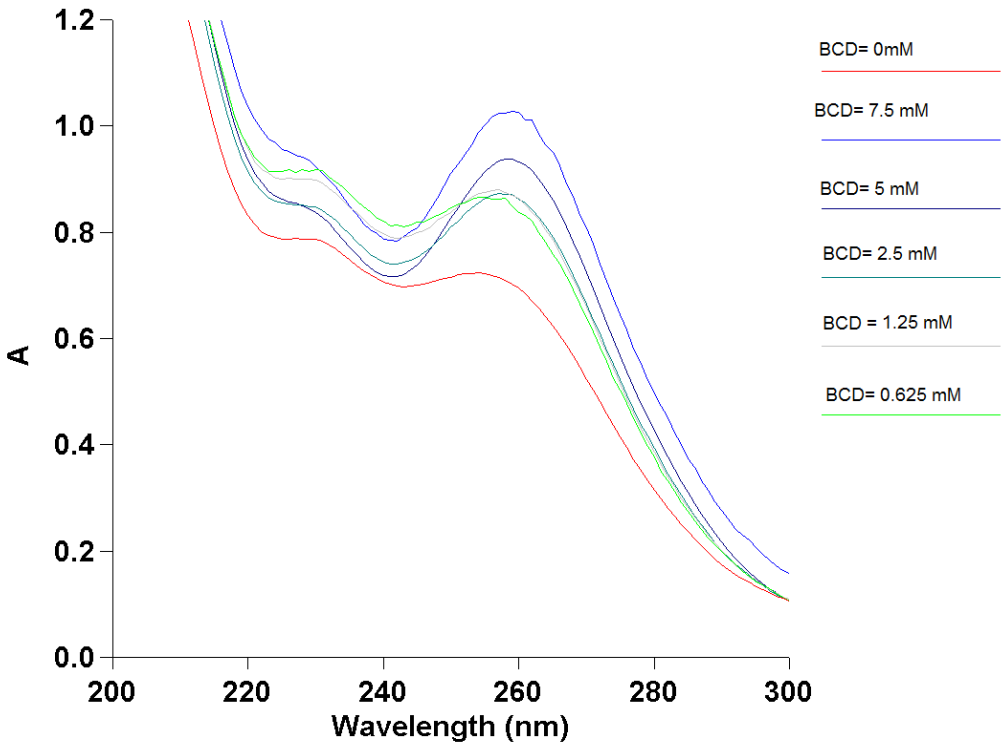

Figure 1B: The shift in the wavelength of the absorbance peak of CHXB from 254nm to 258nm when CHXB is complexed with certain concentrations of BCD. The legend describes the concentration of BCD used. A refers to the absorbance.

The CHXA structure is shown in Figure 2A. It consists of two hydrophobic aromatic groups at the end of the structure and a cationic hydrophilic hexamethylene structure in between. These two hydrophobic aromatic groups complex inside the BCD cavity (Figure 2B). It can also be noted here that the complexation ratio between Chlorhexidine and BCD is 2:1 [6]. As mentioned the complexation is irrespective of whether the Chlorhexidine is in its salt form (CHXA) or base form (CHXB). The solubility of the Chlorhexidine type (CHXA vs CHXB) does not seem to influence the complexation ability with BCD.

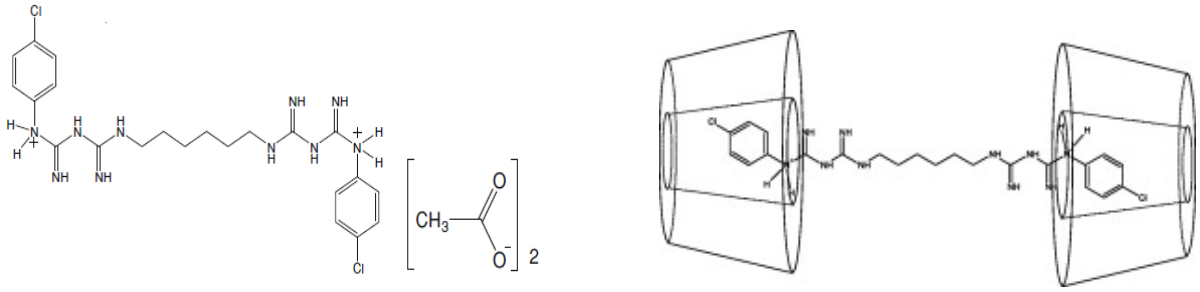

Figure 2A and 2B: A) The chemical structure of Chlorhexidine Acetate (CHXA) and B) Complexation of Chlorhexidine with BCD [6].

It was concluded that the CHXA type of Chlorhexidine would be preferable to exhaust treat cotton for further experiments since it would allow the use of water based liquor due to its higher water solubility. The water solubility of CHXA is about 200 times more than that of CHXB in water. Use of CHXB would require the use of ethanol for the exhaust liquor if a large range of application concentrations were going to be used for further studies. Another disadvantage with the use of ethanol would be the limitations in commercial laundries or industrial pad dry applications where large quantities of ethanol usage would be impractical.

3.2. Loading of CHXA on BCD-Cotton and plain cotton

From the absorbance measurements of the exhaust bath containing the cotton fabric and CHXA, the mass of CHXA on BCD-cotton and cotton could be calculated. From the Figure 3, it is clear that CHXA loading on BCD-cotton and cotton is similar at lower application concentrations. At higher applications, BCD-cotton seems to have higher loading of CHXA than plain cotton. The readings for both types of fabrics show saturation at 2-2.5 g/l of application concentrations.

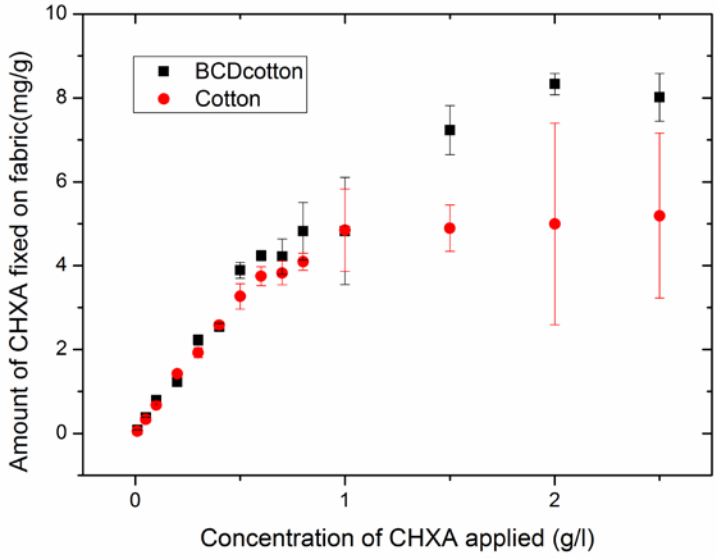

Figure 3: Amount of CHXA loaded onto BCD-cotton and cotton in mg/g of cotton.

It is reported that the cationic biguanide groups of Chlorhexidine have affinity for the anionic carboxyl sites on cotton through electrostatic interaction and Chlorhexidine adsorption on cotton shows a typical Langmuir isotherm at lower application concentrations due to these electrostatic interactions [7]. The contribution of the hydrogen bonding between hydroxyl groups on cotton is said to not contribute significantly to the binding between CHXA and cotton. It appears therefore that with CHXA treatment of BCD-cotton and plain cotton fabrics at lower application concentrations, the CHXA prefers to bind with cotton. At higher application concentrations, additional amount of CHXA is adsorbed onto the BCD-cotton fabric due to the hydrophobic interactions between CHXA and BCD.

3.3. Antibacterial testing

Antibacterial experiments were conducted with CHXA-BCD-cotton and CHXA-cotton fabrics. The antibacterial tests were conducted with ATCC 11229 strain of *E. coli* bacteria according to JIS L 1902 absorption method. The results are expressed in terms of Log CFU or the number of alive bacteria eluted from the fabric after the testing in Figure 4.

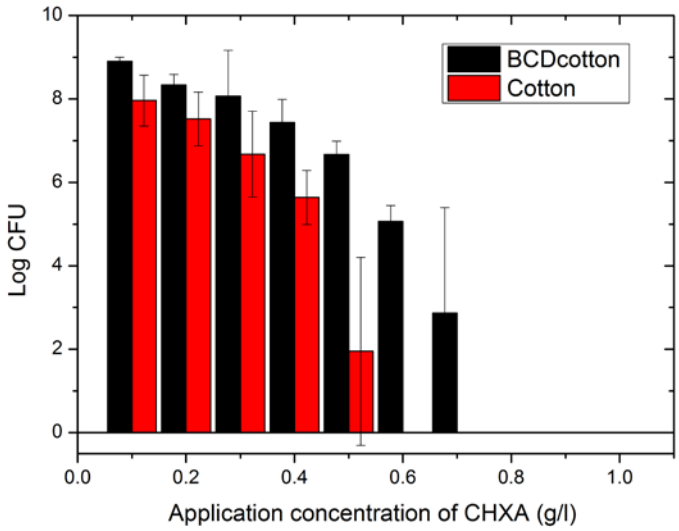

Figure 4: The amount of bacteria eluted (log CFU) from CHXA-BCD-cotton and CHXA-cotton treated with different applications concentrations of CHXA. (CFU = colony forming unit). CHXA-BCD-cotton is shown in the legend as BCDcotton and CHXA-cotton is shown as Cotton.

The antibacterial mechanism of CHXA proceeds with the cationic biguanide groups displacing the anionic groups at the outside of the bacterial cell membrane and disrupting the cellular membrane by solubilizing its phospholipid bilayer to kill the bacteria [3]. From the antibacterial tests, it is clear that CHXA-BCD-cotton shows slow release phenomenon. The amount of bacteria killed on CHXA-cotton is much higher than the CHXA-BCD-cotton as seen in Figure 4. Both these fabrics were treated between 0.1-1 g/l of application concentrations of CHXA and the amount of CHXA on the fabrics (in mg/g) on both these fabrics is nearly the same as seen in Figure 3.

The antibacterial activity calculated from equation (1) is shown in Table 1.

CHXA (g/L)	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**1.0**
Antibacterial Activity BCD-cotton	0.30	0.87	1.30	1.77	2.53	4.13	5.13	6.95	8.43
Antibacterial Activity Cotton	1.25	2.10	2.53	3.56	6.63	7.28	7.55	7.98	9.20

Table 1: Antibacterial activity for CHXA treated BCD-cotton and CHXA treated cotton.

From Table 1 it can be seen that the antibacterial activity for CHXA-cotton is consistently higher than for CHXA-BCD-cotton for the same application concentrations. According to the JIS L 1902 standard, an antibacterial textile should show a minimum of antibacterial activity of two (in log) to be considered antibacterial. The CHXA-cotton samples show the required antibacterial activity from CHXA application concentrations of 0.2 g/l while the CHXA-BCD-cotton samples show antibacterial activity from 0.5 g/l. Though the CHXA-BCD-cotton

shows lower antibacterial activity for the same application concentrations as compared to CHXA treated plain cotton, this can be considered to be significantly advantageous in medical applications.

The complexation of CHXA with BCD-cotton allows protection of CHXA from rapid release. This would mean that instead of rapid killing of bacteria and their rapid bacterial regrowth on textiles, such BCD fabrics would show sustained antibacterial performance with steady killing of bacterial load on the textile and prevention of rapid regrowth of bacteria. This phenomenon would be useful in every day clothing where prolonged bacteriostatic effect is preferable over bactericidal property for a short duration. It is also useful in wound dressings where due to the slow release phenomenon there is less frequent change of wound dressings. Such sustained release dressings are known to encourage better healing and wound repair [8]. This type of slow release phenomenon would also reduce the risk of local cellular toxicity of antimicrobial agents.

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