Use of Natural Polysaccharides in Medical Textile Applications

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To my parents, my brothers, my wife

Abstract

Polysaccharides are composed of many monosaccharide units that are joined one to the other by an acetal linkage to give a long chain. Many natural polysaccharides and oligosaccharides participate in variety of biochemical reactions *in vivo*. However, it is quite difficult to elucidate the mechanism of the biological activity of the saccharide chain because of its complex chemical structure and impurities. In this work two special polysaccharides have been used as candidates in medical textile applications: chitosan and ß-cyclodextrin.

Chitosan is linear polysaccharide consisting of ß- (1,4)-2-amino-2-deoxy-D-glucopyranose units. It is produced by alkaline deacetylation of chitin. Today, chitin and chitosan are known as novel materials used in the biomedical, pharmacological and biotechnological fields. In this thesis chitosan of different molecular weight (MW) and origin has been prepared and characterized; MW and degree of deacetylation (DD) using Gel Permeation Chromatography (GPC) and hydrochloric acid back titration, respectively. The antimicrobial activity of chitosan has been measured by using different and new methods such as tetrazolium/formazan test (TTC) and laser nephelometry in microtiter plate, taking in account the different factors affecting this antimicrobial property such as MW, pH and temperature. Carboxymethyl chitosan (CMCTS) has been synthesized from chitosan and monochloroacetic acid in the presence of alkali. The product is water soluble. Chitosan and its derivative (CMCTS) have been used as a new finishing agent in medical textile application. Different anchors and methods have been used to fix chitosan and its derivative on to cotton fabrics forming chemical bonds. The physical properties and dyeing performance of the finished fabrics have been evaluated, in addition the antibacterial activity of the treated cotton fabrics has been measured using the TTC-test method.

Chitosan also in this thesis has been used as antimicrobial coating agent in modern dentistry to treat denture stomatitis. Denture stomatitis is the most common form of oral candidosis. Different types and MW of chitosan films have be prepared on Molloplast® B soft liner denture based materials. These denture soft liners are viscoelastic materials used to form all or part of the fit surface of the denture which is commonly used for elderly patients in order to relieve pain and more comfort for them. In this thesis we focus our interest on pathways to develop potentially biocompatible soft den-

ture liners coated with chitosan films that need not to be removed and of course can deliver medication to treat this disease called denture stomatitis.

Cyclodextrins (CDs) are cyclic oligosaccharides that are produced by the enzymatic degradation of starch. Three different CDs can be obtained in various proportions: a-, ß-, and γ–CDs constituted of six, seven and eight glucopyranose units respectively. The three natural CDs differ not only in their diameter, related to the number of glucopyranose units, but also in their water solubility. One of the most important characteristics of CDs is the formation of inclusion complexes with various guests in which the guest molecules are included in the hydrophobic cavities of CDs (host). These inclusion complexes have new physicochemical properties, stability and apparent solubility, resulting in new pharmacotechnical characteristics and consequently, better therapeutic efficiency. In this thesis ß-cyclodextrin (ß-CD) has been used as a drug release candidate in pharmaceutical application. The very low aqueous solubility of both antifungal agents (econazole nitrate and ciclopirox olamine) does not allow the preparation of a concentrated stock solution. Therefore, complexation with ß-CD has been improved the aqueous solubilities of both drugs without modification of their original structures. This may allow a homogenous delivery system of both antifungal agents, and hence increase their bioavailability. The antimycotic influence of ß-cyclodextrin complexes against *Candida albicans* DSM 11225 and *Candida krusei* ATCC 6258, hence, the drug solubility has been measured using a new, rapid and novel method called “laser nephelometry in microtiter plates”.

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List of Abbreviations

ATCC	American Type Culture Collection
BTCA	1,2,3,4-Butanetetracarboxylic acid
ß-CD	ß-Cyclodextrin
Cfu	Colony forming unit
CNC	Cyanuric chloride
CTS	Chitosan
CMCTS	Carboxymethyl chitosan
cN	Centi Newton
COS	Chito-oligosaccharide
CO	Cotton
CRA	Crease recovery angle
CGTase	Cycloglycosyl transferase
CDs	Cyclodextrins
CI	Ciclopirox olamine, (6-cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridone)
CD-EC	ß-Cyclodextrin-econazole nitrate complex
CD-CI	ß-Cyclodextrin-ciclopirox olamine complex
Da	Dalton
DC	Degree of carboxylation
DD	Degree of deacetylation
DP	Degree of polymerisation
DQ	Degree of quaternization
DS	Degree of substitution
DSC	Differential scanning calorimetry
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTNW	Deutsches Textilforschungszentrum Nord-West e.V.
E	Elongation at break
EC	Econazole nitrate (1-[2-(2,4) dichlorophenyl)-2-

	(4-chlorobenzyloxy)-ethyl]-imidazole nitrate)
FUN®-1	Fluorescence marker stain for fungi
G	Gram
GPC	Gel permeation chromatography
IC	Inclusion complex
MAA	Methacrylic acid
MCNC	2,4-Dichloro-6-methoxy-s-triazine
MIC	Minimum inhibitory concentration
MW	Molecular weight
MWD	Molecular weight distributions
PA	Polyamide
PES	Polyester
PAN	Polyacrylonitrile
PBS	Phosphate buffered saline
PTCA	Propane tricarboxylic acid
PESNa	Poly-ethylensulfonic acid, sodium salt
PDADMAC	Poly-di-allyl-di-methyl-ammonium-chloride
Quab® 151	2,3-Epoxypropyltrimethyl ammonium chloride
SI	Standard I (nutrient broth medium for bacteria)
SGB	Sabouraud-Glucose-Bouillon
SGA	Sabouraud-Glucose-Agar
TTC	2,3,5- Triphenyltetrazolium chloride
TS	Tensile strength
WO	Wool
WPU	Wet pick-up

Chapter 1

Introduction

1.1. Medical textiles

Medical textiles account for a huge market due to widespread and not only in hospitals, hygiene and healthcare sectors but also in hotels and other environments where hygiene is required. There has been a sharp increase in the use of natural as well as synthetic fibres in producing various medical products. The annual growth of medical textile products is likely to be around 10 % during the year 1999-2000 [1].

It is known that micro organisms create and aggravate problems in hospitals and other environments by transmitting diseases and infections through clothing, bedding etc. The axillae and perineal regions of the body are more susceptible to microbial growth that leads to undesirable body odour. It is reported that polyamide fibres retain more odour causing micro organisms than natural fibres [2]. Polyesters and other synthetic fibres are also subjected to the growth of pathogenic micro organisms. Besides micro organisms deteriorate cellulosic fibres and reduce the wear life of the materials [3]. They adhere to the surface of the fibres, gradually corrode inwards layer by layer disintegrating the primary and secondary walls of the fibres causing considerable damage [4]. It may be noted that bacteria are usually active at pH 7.0-8.0 and fungi at 4.0-6.5. A large number of fungi have been isolated on exposed cotton textiles [5]. Thus micro organisms exist in abundant quantities on textile materials. In order to combat these adversities, it is highly desirable to impart antibacterial, antifungal and mildew resistance properties to textile materials.

With a view to develop such textile materials, considerable research has been carried out by making use of organic and inorganic compounds, antibiotics, heterocyclic, quaternary ammonium compounds and so on. Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol is an important bactericide example used in various personal care (shampoo, toilet soap, deodorants, tooth paste) and consumer (footwear, plastic wear) products [6]. Some recent developments in antibacterial products include a process involving the preparation of antibacterial resins containing phenol derivatives [7]. The resins exhibited higher antibacterial activity against *Escherichia coli* and

Staphylococcus aureus. Beliakova et al. discussed the process for preparing an antibacterial formulation by reaction of carboxymethyl starch with trimethylolated melamine in the presence or absence of cupric ions to render cotton fabric antibacterial [8]. The cellulose has been modified chemically with biocides accompanied by redox reaction to achieve durable and regenerable antibacterial activity on cotton and other cellulosic fabrics [9]. It is well known that many infectious diseases can be prevented or sterilized by wearing antibacterial fabrics-protective clothing. Burn patients and people who do not have functioning immune systems also need to wear these germ-free or germicidal textiles to avoid infection.

Antimicrobial materials such as fabrics, polymers and even toys are becoming more and more popular due to the public concerns on pathogens [10]. These antimicrobial materials can be divided into two categories based on the ability against micro organisms, i.e., biocide and biostatic functions. Biocide functions refer to the inactivation of micro organisms on the materials or total kill, while biostatic properties indicate the inhibition of the of micro organisms growth by the materials or partial kill. Biostatic fabrics would be more appropriate for aesthetic and hygienic type applications of textile products.

Today, with increasing awareness of environmental concerns, a significant amount of legislation on ecotoxicological considerations has been introduced [11]. Holme has indicated the challenges faced, and accordingly the changes required in functional finishes for cotton fabrics [12]. The major factors that will act as a driving force for change in chemical finishing for cotton in the next decade are the following:

- The need for better quality and higher added value chemical finishes for cotton,
- More environmental-friendly chemical finishes and application methods,
- Increased possibility of process integration to minimize the use of water and energy,
- Increased levels of process control, monitoring in textile finishing machinery.

1.2. Objectives of this thesis

As mentioned in the previous section, this thesis falls into two distantly related parts. Both of the two parts investigate in details the use of natural polysaccharides in medical textile applications. The first part discusses chitosan, its antimicrobial properties, and different methods for evaluation of its antimicrobial property. Also, from this point of view, chitosan and its derivatives will be used and applied as antimicrobial finishing agent in textile finishing application. Chitosan will be finally used as antimicrobial coating agent in the field of modern dentistry in order to treat the so called oral denture stomatitis. The second part will discuss ß-cyclodextrin as an exemplary drug delivery cyclic carbohydrate and its application in medical textiles. The following aspects have been worked on and the results presented below have been achieved.

1.3. Chitosan

There is a greater demand for antimicrobial finishes on textile goods because consumers have become aware of the potential advantages of these materials. A number of other chemicals are also used in textile processes. Many of these chemicals, however, are toxic to humans and do not easily degrade in the environment. The textile industry continues to look for eco-friendly substitutes for toxic chemicals and to reduce dyes in works wastewater. These aims have facilitated the use of chitosan (CTS) as a new textile chemical. CTS, an important and commercially available biopolymer, has many chemical attributes to make it an interesting candidate for these applications.

Chitosan is the deacetylated derivative of chitin, which is the second most abundant polysaccharide found on earth next to cellulose. Chitin is the main component in the shells of crustaceans, such as shrimp, crab and lobster. It is also found in exoskeletons of insects and in the cell walls of some fungi [13]. In the past 30 years, it has been demonstrated by a number of researchers that chitosan has a great potential for a wide range of uses due to its biodegradability, biocompatibility, antimicrobial activity, non-toxicity, and versatile chemical and physical properties. The applications of CTS include uses in a variety of areas, such as pharmaceutical, medical applications, paper production, wastewater treatment, biotechnology, cosmetics, food processing and agriculture [13-17].

1.3.1. Chemical structure

Chitosan is a linear polysaccharide consisting of ß- (1-4)-2- amino -2- deoxy-D-glucopyranose units and its idealised structure is similar to cellulose as shown in Figure 1. Chitin has the same backbone as cellulose, but it has an acetamide group on the C-2 position instead of a hydroxyl group and its molecular weight, purity and crystal morphology are dependent on its source [18]. CTS is the *N*-deacetylated derivative of chitin, and most of its glucopyranose residues are 2-amino-2-deoxy-ß-D-glucopyranose.

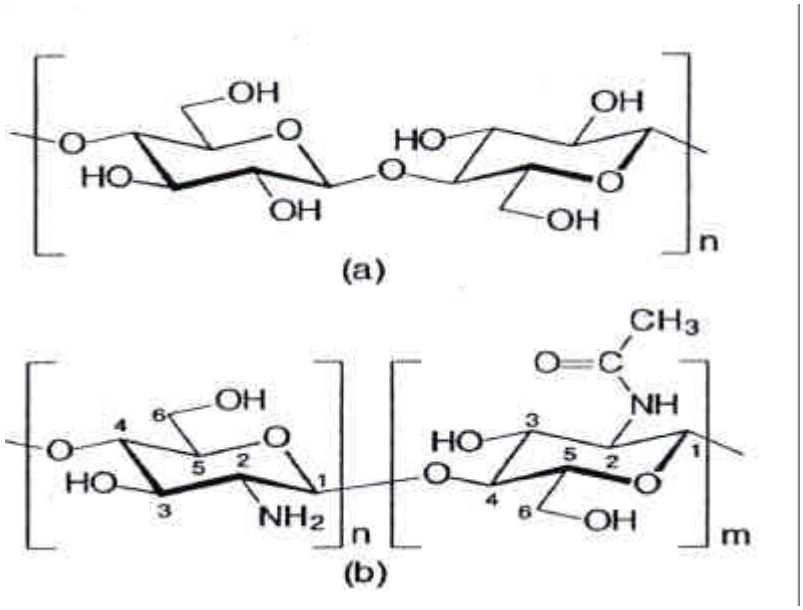


Figure 1: Chemical structures of (a) cellulose and (b) chitin and chitosan (chitin occurs mostly the “m” or *N*-acetyl form and chitosan occurs as the “n” or amino form)

1.3.2. Preparation of chitosan

Chitosan is commonly prepared by deacetylating a-chitin using 40-50 % aqueous alkali at 100-160 °C for a few hours as described in Figure 2 [19]. The resulting chitosan has a degree of deacetylation (DD) up to 0.95. For complete deacetylation, the alkaline treatment can be repeated. Complete deacetylation is rarely achieved if it is necessary. The solubility in dilute aqueous acids is obtained at an extent of deacetylation of = 60 %.

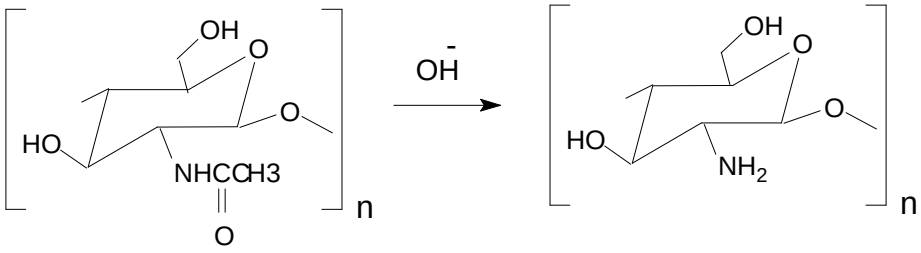


Figure 2: Preparation of chitosan

1.3.3. Properties of chitosan

Most of the naturally occurring polysaccharides, e.g. cellulose, dextran, pectin, alginic acid, agar, agarose and carrageenan, are neutral or acidic in nature, whereas chitin and chitosan (CTS) are examples of highly basic polysaccharides. Their unique properties include polyoxysalt formation, ability to form films, chelate metal ions and optical structural characteristics [20].

Like cellulose, chitin functions naturally as a structural polysaccharide, but differs from cellulose in its properties. Chitin is highly hydrophobic and insoluble in water and most organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone and chloroalcohols in conjugation with aqueous solutions of mineral acids [21] and dimethylacetamide containing 5 % lithium chloride. CTS, the deacetylated product of chitin, is soluble in dilute acids such as acetic acid, formic acid, etc. The hydrolysis of chitin with concentrated acids under drastic conditions produces relatively pure D-glucosamine.

The nitrogen content of chitin varies from 5 to 8 % depending on the extent of deacetylation, whereas the nitrogen in CTS is mostly in the form of primary aliphatic amino groups. CTS, therefore, undergoes reactions typical of amines, of which *N*-acylation and Schiff reaction are the most important.

Chitosan is an abundant natural resource that has, as yet, not been fully utilized. Advantages of this biopolymer include availability, medium cost, high biocompatibility, biodegradability and ease of chemical modification [22].

1.3.4. Solubility of chitosan

Chitosan, being a base, forms salts with acids so producing polyelectrolyte whose solubility's will depend on the nature of the anion involved. Salt formation may take place as a separate step, followed by addition of the CTS salt to water but the more common practice is to add acid to CTS suspended in water so that salt formation and dissolution occur concurrently [23]. In those cases where the CTS salt has limited aqueous solubility other factors such as the molecular weight and degree of *N*-acetylation of the CTS, the total amount of acid present and the temperature, become important and it is not surprising that there are some conflicting reports in the literature in respect of the solubilities of some chitosan salts.

The solubility of CTS is very important for its utilization, such as for chemical modification and film or fibre formation. Both chitin and chitosan are not soluble in neutral water. Chitin is a semicrystalline polymer with extensive inter and intramolecular hydrogen bonds, which make it difficult to dissolve in dilute acids or organic solvents under mild conditions. Although many solvents have been found, but most of them are not useful due to their toxicity, corrosiveness, or mutagenic properties. CTS readily dissolves in dilute mineral or organic acids by protonation of free amino groups at pH below about 6.5. This cationic nature is the basis of a number of applications of CTS. Acetic and formic acids are widely used for research and applications of CTS. Some of the organic acids which can dissolve CTS are listed in Table 1. On the other hand, CTS is insoluble in dibasic mineral acids as H_2SO_4. Generally, the solubility of chitin and chitosan decreases with an increase in MW. Oligomers of chitin and CTS with degree of polymerization (DP) of 8 or less are water soluble regardless of pH [24].

	Concentration of chitosan used				
Acids	1 %	5 %	10 %	50 %	> 50 %
Acetic	+	+	+		
Adipic	+				
Citric	-	+	+		
Formic	+	+	+	+	+
Lactic	+	+	+		
Malic	+	+	+		
Malonic	+	+	+		
Oxalic	+		+		
Propionic	+	+	+	+	
Succinic	+	+	+		
Tartaric	-		+		

Table 1: Solubility of chitosan in organic acids [25] (+, soluble; -, insoluble)

1.3.5. Solution properties of chitosan

In acid solution the amine groups of chitosan (CTS) are protonated and under these conditions CTS would be expected to exhibit behaviour typical of a polyelectrolyte. Van Duin and Hermans [26] and Kienzle-Sterzer et al. [27] showed that in dilute solutions in the absence of added electrolyte there is an abnormal increase in the viscosity number with decrease in CTS concentration, but that normal straight line viscosity number versus concentration plots are obtained in the presence of added electrolyte. This is typical of polyelectrolyte solutions and is a result of the increase in coil dimensions that occurs on dilution owing to electrostatic repulsion between chain segments. This repulsion may be suppressed by adding a low molecular weight (low-MW) electrolyte which functions by screening the electrical charges on the polymer chain. The viscosity number has also been found to decrease with increasing acetic acid concentration at a given CTS concentration [27].

1.3.6. Chemical modification of chitosan

Chitosan (CTS) has three reactive groups, i.e., primary (C-6) and secondary (C-3) hydroxyl groups on each repeat unit, and the amino (C-2) group on each deacetylated unit as illustrated in Figure 3. These reactive groups are readily subjected to chemical modification to alter mechanical and physical properties and solubilities of CTS. The typical reactions involving the hydroxyl groups are etherification and esterification. Selective *O*-substitution can be achieved by protecting the amino group during the reaction. As *N*-protected CTS derivatives, several Schiff bases of CTS and *N*-phthaloyl chitosan have been reported [28-30]. The presence of a nucleophilic amino group allows selective *N*-substitution, such as *N*-alkylation and *N*-acylation by reacting CTS with alkyl halides and acid chlorides, respectively. The alternative method for the *N*- alkylation is reductive alkylation, where the amino group is converted to an imine with a variety of aldehydes or ketones and subsequently reduced to a *N*-alkylated derivative. The presence of the more or less bulky substituent weakens the hydrogen bonds of CTS; therefore *N*-alkyl chitosans swell in water in spite of the hydrophobicity of the alkyl chains, but they retain the film forming property of CTS [31]. Also CTS can be modified by either cross-linking or graft copolymerization. A number of modified CTS derivatives are listed in the literature [32,33].

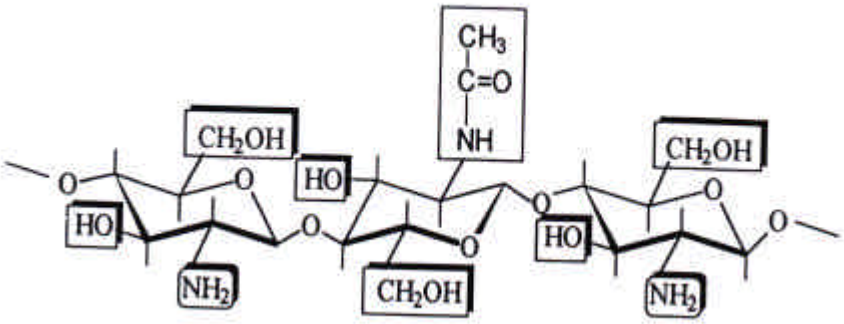


Figure 3: Possible reaction sites of chemical modification of chitin and chitosan

1.3.7. Characterization of chitosan

The structural details of cellulose, chitin and chitosan (CTS) have been shown in Figure 1. Cellulose is a homopolymer, while chitin and CTS are heteropolymers. Neither random nor block orientation is meant to be implied for chitin and CTS. The properties of chitin and CTS such as the origin of the material (discussed in a previous section), the degree of *N*-deacetylation and molecular weight are discussed in brief.

Degree of *N*-deacetylation

An important parameter to examine closely is the degree of *N*-acetylation in chitin, i.e. the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose structural units. This ratio has a striking effect on solubility and solution properties. Chitin does not dissolve in dilute acetic acid. But when chitin is deacetylated to a certain degree (approximately 60 % deacetylation) where it becomes soluble in acid, it is referred to as chitosan. In chitin, the acetylated units prevail (degree of acetylation typically 0.9). CTS is the fully or partially *N*-deacetylated derivative of chitin with a typical degree of acetylation less than 0.4. To define this ratio, a number of methods have been used, such as FTIR spectroscopy [34], UV spectroscopy [35], ^{1}H NMR spectroscopy [36], ^{13}C solid-state NMR spectroscopy [37], gel permeation chromatography [35], titration methods [37-39], equilibrium dye adsorption [40], elemental analysis [41], acid degradation followed by HPLC [41] and thermal analysis [42].

Molecular weight (MW)

Molecular weight (MW) is a very important parameter for the application of natural and synthetic polymers. The MW of chitin and chitosan depend on its source and deacetylation conditions (time, temperature and concentration of NaOH), respectively. CTS obtained from deacetylation of chitin may have a MW over 100.000. Consequently, it is necessary to reduce the MW by chemical methods to a much lower MW for easy application as a textile finish. The MW of CTS can be determined by several methods, such as light scattering spectrophotometry [43], gel permeation chromatography and viscometry [44].

Viscometry is one of the simplest and most rapid methods for determining the molecular weights of polymers, despite having the disadvantage of not being an absolute method since it requires the determination of constants through correlation of limiting viscosity number (LVN) values with MW values measured by an absolute method [32]. The most commonly used equation relating LVN values to MW is the Mark-Houwink equation:

$$[\eta] = K \cdot MW^{\alpha}$$

where $[\eta]$ is the LVN and K , α are constants that are independent of MW over a considerable range of MW. They are dependent on polymer, solvent, temperature and, in case of polyelectrolytes, the nature and concentration of the added low - molecular - weight electrolyte. The constants are normally evaluated from a plot of log $[\eta]$ versus log MW for a series of carefully prepared fractions having very narrow molecular weight distribution (MWD) values. Several sets of values for K and α have been proposed for chitosan in the literature and these are given in Table 2. The units used for K in Table 2 are $cm^3\ g^{-1}$ and the literature values have been converted to these units from $dl\ g^{-1}$ where necessary. The solvent systems used and MW ranges over which the constants were determined in each case are also given. We considered that one important reason for the difference in K and α was the degree of deacetylation (DD) of the chitosan samples used. As is known, chitosans behave as a form of polyelectrolyte in dilute acid aqueous solution. The linear charge density along the chain increases with increase in the DD and this might be expected to give rise to a gradual increase in intrinsic viscosity due to the coil expansion. The values of K and α in the Mark-Houwink equation might also change with the DD of chitosan. Wang et al. determined the viscometric constants K and α in the Mark-Houwink equation using chitosan with different DD by the light scattering method [45]. They concluded that the values obtained for K and α are not constant if the DD of chitosan changes. In this thesis the MW will be determined by gel permeation chromatography (GPC). Details about the method and the system will be discussed later in chapter 2.

Solvent system	K ($cm^3 g^{-1}$)	a	MW-range (10^5)
1. 0.2 M HOAc/0.1 M NaCl/4 M urea	8.93×10^{-2}	0.710	1.13-4.92
2. 0.167 M HOAc/0.47 M NaCl	111.5	0.147	0.13-1.70
3. 0.1 M HOAc/0.2 M NaCl	1.81×10^{-3}	0.930	0.90-11.4
4. 0.33 M HOAc/0.3 M NaCl	3.41×10^{-3}	1.020	0.13-1.35
5. 0.33 M HOAc/0.2 M NaOAc/ 0.67 M $ClCH_2COOH$	1.28×10^{-2}	0.850	0.61-1.60

Table 2: Values of the Mark-Houwink equation constants K and a for chitosan according to [32]

1.3.8. Medical uses

Chitosan (CTS) is currently receiving a great deal of interest for medical and pharmaceutical applications. Some of these applications are summarized in Table 3. The main reasons for this increasing attention are certainly its interesting intrinsic properties. Indeed, CTS is known for being biocompatible allowing its use in various medical applications such as topical ocular application, implantation or injection [46-48]. Due to its positive charges at physiological pH, CTS is also bio adhesive, which increases retention at the site of application [49]. CTS also promotes wound-healing and has bacteriostatic effects [50,51]. Finally, CTS is very abundant and it has medium cost production and is ecologically interesting [52]. In medical and pharmaceutical applications, chitosan is used as a component in hydrogels.

Chitosan has been used for the first time in dentistry [53], on a total of 24 patients, all of which recovered completely. Neither allergic reactions nor infections took place. CTS could be used as a transparent membrane or as a thin powder, soaked in antibiotic solution; it accelerates wound healing and promotes regular fibrin formation [53]. CTS ascorbate was used for the reduction of the periodontal pockets in the surgical interventions, and the evaluation of its clinical and morphostructural efficiency in the reconstruction of the periodontium [54]. In this thesis CTS was used as antimicrobial coating agent in modern dentistry to treat denture stomatitis [55].

Physical form	Application
Solution	Bacteriostatic agent
	Haemostatic agent
	Cosmetics
Gel	Delivery vehicle
	Spermicide
Powder	Surgical glove powder
	Enzyme immobilization
Film/membrane	Dialysis membrane
	Contact lens
	Wound dressing
Sponge	Mucosal haemostatic dressing
	Wound dressing
Fibres	Improvement of dyeability
Miscellaneous	Anticholesteremic materials
	Antigastritis agents
	Anticoagulants

Table 3: Biomedical applications of chitosan [25]

1.3.9. Antimicrobial activity

The antimicrobial activity of chitosan against various bacteria and fungi is well known, and it has been reported by a number of authors [51,56-60]. In this section, mechanisms of this action and factors affecting antimicrobial activity are described.

Mechanism

Several different mechanisms for microbial inhibition by chitosan (CTS) have been proposed, but the exact mechanism is still not known. The most accepted one is the interaction of the positively charged CTS with the negatively charged residues at the cell surface of many fungi and bacteria, which causes extensive cell surface alterations and alters cell wall permeability [56,58-61]. This causes the leakage of intra-

cellular substances, such as electrolytes, UV-absorbing material, proteins, amino acids, glucose, and lactate dehydrogenase. As a result, CTS inhibits the normal metabolism of micro organisms and finally leads to the death of these cells. For example, Fang et al. reported that the growth of *Aspergillus niger* was inhibited by chitosan [58]. CTS at the concentration of 5.0 mg/ml induced considerable leakage of UV-absorbing and proteinaceous materials from *A.niger* at pH 4.8. In contrast, CTS at pH 7.6 and chitin at pH 4.8 did not induce leakage, which suggests that the antifugal activity of CTS is related to the polycationic nature of CTS and is directly affected by the pH value. The leakage of nucleic acid and protein from *Escherichia coli* was observed by Hwang et al. in their study on the bacterial activity of chitosan on *E.coli* [61]. Transmission electron microscopy (TEM) revealed that the outer cell wall of *E.coli* was greatly distorted and frayed, and the cytoplasmic membrane was detached from the inner part of the cell wall after CTS treatment. Tsai and Su observed the chitosan-induced leakage of glucose and lactate dehydrogenase from *E.coli* cells and suggested that the death of the cells resulted from the interaction between CTS and the *E.coli* cell, and change in membrane permeability, which cause the leakage of intracellular components, such as glucose and lactate dehydrogenase [59].

Another mechanism proposed that the positively charged CTS interacts with cellular DNA of some fungi and bacteria, which consequently inhibits the RNA and protein synthesis [51,62]. In this mechanism, CTS must be hydrolyzed to a lower MW to penetrate into the cell of micro organisms. However, this mechanism is still controversial. Tokura et al. examined the antimicrobial action of CTS with weight average MW of 2200 and 9300 having DD of 0.54 and 0.51, respectively [63]. It was observed that the chitosan of MW 9300 was stacked on the cell wall and inhibited the growth of *E. coli*. However, CTS of MW 2200, which permeated into the cell wall, accelerated the growth of *E.coli*. They suggested that the antimicrobial action is related to the suppression of the metabolic activity of the bacteria by blocking nutrient permeation through the cell wall rather than the inhibition of transcription from DNA.

Factors affecting antimicrobial activity

The extent of the antimicrobial action of chitosan is influenced by intrinsic and extrinsic factors such as MW, DD, pH, temperature etc. It is necessary to understand these factors for the effective application of chitosan as an antimicrobial agent.

Molecular weight (MW)

Tanigawa et al. reported that *D*-glucosamine hydrochloride (chitosan monomer) does not show any growth inhibition against several bacteria, whereas CTS was effective [64]. This suggests that the antimicrobial activity of CTS is related to not only its cationic nature but also its chain length. Hirano and Nagao examined the relationship between the degree of polymerisation (DP) of CTS and the growth inhibition of several phytopathogens [65]. Three different MW chitosan, high MW (HMW) CTS (MW 400.000, DD = 0.95), low MW (LMW) CTS (MW and DD :not specified) and CTS oligomer (DP 2-8) were used. The strongest growth inhibition was observed with LMW-chitosan and the weakest was observed with HMW-chitosan. This was explained by the difficulty of the HMW-chitosan to diffuse into the agar gel containing the test organism due to its high viscosity.

Shimojoh et al. also suggested that the antimicrobial activity is heavily dependent on the MW of CTS [66]. Several oral bacteria were treated with the same concentration of chitosans with four different MW (DD = 0.99) from squid chitin (ß-chitin) in 1 % lactate buffer (pH 5.8) for 1 min and incubated at 37 °C for 24 h. It was found that the chitosan with MW 220,000 was most effective and MW 10,000 was the least effective in their bactericidal activities. The antimicrobial activities of chitosans with MW of 70.000 was better than with MW 426.000 for some bacteria, but for the others, the effectiveness was reversed. This finding suggests that the antimicrobial activity of chitosan varies depending on the target micro organisms.

Yalpani et al. reported that chitosans (medium and high MW) showed higher antimicrobial activities against *Bacillus circulans* than chito-oligosaccharides (DP 2-30), whereas they were less effective against *E.coli* than chito-oligosaccharides [67]. From the results of Shimojoh [66] and Yalpani [67], one can notice that the relationship between MW of CTS and the antimicrobial activity can be affected by the test organisms.

Numerous researchers have reported the antibacterial activity of CTS against *E.coli* [51,61,64,68]. Hwang et al. concluded that CTS with MW about 30,000 exhibited the highest bactericidal effect on *E.coli* from their investigation of CTS with a MW range of 10.000-170.000 [61]. Jeon et al. suggested that the MW of CTS is critical for the inhibition of micro organisms and suggested the required MW be higher than 10.000 for better antimicrobial activity [68].

To elucidate the relationship between MW of CTS and its antimicrobial activity against *E.coli*, the existing experimental data are summarized in Table 4. Although it is difficult to find a clear correlation between MW and antimicrobial activity, generally the antimicrobial activity increases as the MW of chitosan increases. However, the activity decreases above a certain high MW. The discrepancies between data may result from the different DD and MWD of CTS. The evaluation of only the MW dependence of antimicrobial activity requires a wide MW range of CTS samples with the same DD and MWD. It is almost impossible to obtain this because CTS is a natural polymer. There is always variation from batch to batch, and the properties of CTS are very sensitive to DD and molecular weight distributions (MWD). Therefore, from the existing data, it is difficult to determine what the most optimal MW for the maximal antimicrobial activity is. The selection of MW of CTS could be thought to be more dependent on its application.

References	Effectiveness, MW (DD)
Hwang	29,800 (0.93) > 102,200 (0.93) > 9,800 (0.96) > 174,700 (0.94)
Liu	91,600 (0.86) > 51,100 (0.88) > 8,000 (0.75) > 5,000 (0.73), 274,000 (0.74) > 650,000 (0.85), 1,080,000 (0.85)
Jeon	685,000 (0.89) ≥ 24,000 – 7,000 > 6,000 – 1,500 > ~ 1,000
Ueno	10,500, 9,300 > 8,000, 7,300 > 6,200 > 5,500 > 4,100, 2,200
Tanigawa	80,000 (0.80) > 166,000 (0.91) > 190,000 (0.84) > 2,000 (< 0.80) > 4,000 (< 0.80) > 12,000, 8,000 (< 0.80)
Chang	35,000 > 29,000 > 32,000 > 97,000, 95,000, 68,000 > 293,000, 275,000 > 820,000, 11,000

Table 4: Effect of chitosan MW on its antimicrobial activity against *E.coli*

Degree of deacetylation (DD)

According to several authors, the antimicrobial activity of chitosan (CTS) is directly proportional to the DD of CTS [51,64,66]. The increase in DD means an increased number of amino groups on CTS. As a result, CTS has an increased number of protonated amino groups in an acidic condition and dissolves in water completely, which leads to an increased chance of interaction between CTS and negatively charged cell walls of micro organisms in solution.

The pH-value

The antimicrobial activity of CTS is strongly affected by pH [51,56,57,59]. Tsai and Su examined the antimicrobial activity of CTS (DD 0.98) against *E.coli* at different pH values of 5.0, 6.0, 7.0, 8.0 and 9.0 [59]. The greatest activity was observed at pH 5.0. The activity decreased as the pH increased, and CTS had little antibacterial activity at pH 9.0. Other researchers reported that CTS had no antimicrobial activity at pH 7.0 due to the deprotonation of amino groups and poor solubility in water at pH 7.0 [51,56]. This finding suggests that the antimicrobial activity of CTS is based on its cationic nature.

Temperature

Tsai and Su examined the effect of temperature on the antimicrobial activity of CTS against *E.coli* [59]. The cell suspensions in phosphate buffer (pH 6.0) containing 150 ppm chitosan were incubated at 4, 15, 25, and 37 °C for various time intervals, and the surviving cells were counted. The antimicrobial activity was found to be directly proportional to the temperature. At temperatures of 25 °C and 37 °C, the *E.coli* cells were completely killed within 5 h and 1 h, respectively. However, at lower temperatures (4 °C and 15 °C), the number of *E.coli* declined within the first 5 h and then stabilized. The authors concluded that the reduced antimicrobial activity resulted from the decreased rate of interaction between CTS and cells at a lower temperature.

Cations and polyanions

Young and Kauss reported that chitosan caused the release of Ca^{2+} present on *Glycine max* cell and/or plasma membrane, which destabilized the cell membrane and further induced leakage of intracellular electrolytes [69]. They suggested that the cross-linking of CTS (polycation) with phospholipids or protein components in the cell membrane affects the membrane permeability, which further causes leakage of intracellular substances, and finally causes the death of the cell. Young et al. observed that chitosan-induced leakage of UV-absorbing material from *G.max* was strongly inhibited by divalent cations in the order of $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Na^{+} > K^{+}$ [70]. It was assumed that the cations displaced Ca^{2+} released from the cell surface, from complexes stabilizing the cell membrane, and consequently reduce the chitosan-induced leakage. Tsai and Su also reported reduced bactericidal effect of CTS against *E.coli* by the addition of salts containing alkaline earth metals such as $MgCl_2$, $BaCl_2$ and $CaCl_2$ [59]. The order of effectiveness was $Ba^{2+} > Ca^{2+} > Mg^{2+}$. The authors proposed that the cations from complexes with chitosan and, consequently, the reduced available amino groups of CTS led to the reduced bactericidal effect unlike Young´s assumption [70].

In addition to the reduced chitosan-induced leakage by cations, the leakage was also reduced by the addition of polyanions such as Na-polygalacturonate and Na-poly-L-aspartate [70]. The complete prevention of leakage of electrolytes was observed when the number of carboxyl groups in the polyanions was equaled to that of the amino groups of CTS. It was attributed to the formation of polycation (chitosan)-polyanion complexes, which were observed by formation of precipitate. However, monomeric galacturonate and aspartate did not show any effect on the leakage, and no precipitation of CTS was observed. The explanation of this provided by the authors was that individual ionic bonds between anionic monomers and polycations could dissociate, but the multiple bonds between polyanion and polycation would not dissociate at the same time.

1.4. Cyclodextrins

The first reference to cyclodextrins (CDs) was published in 1891 [71]. Some years later Schardinger also observed the formation of CDs [72]. At this time nothing was known about the structure of these molecules. Freudenberg continued to study these compounds originally obtained from starch [73]. He called them Schardinger dextrins. Further studies by him and Borchert showed the cyclic structure of Schardinger dextrins [74,75]. From this time they were also called " cyclodextrins". Cramer realized that these CDs were able to include neutral molecules within their cavities [76]. From this time the interest in CDs increased. However CDs were only available in small quantities. Thus no practical applications seemed to be suitable for these molecules. However in 1980 Saenger published a review article about CDs in which he already mentioned some industrial applications [77]. The first international CD symposium organized by Szejtli took place in Budapest (1981) [78]. One year later the first CD book written by Szejtli was published [79]. Since this time an increasing interest in CDs and their possible applications exists. Up to now mainly pharmaceutical applications have been described in literature [80,81]. Some further applications in analytical chemistry, food and very recently textiles are known [82-84].

1.4.1. Nature and characteristics of CDs

Cyclodextrins are produced by the enzymatic degradation of starch [85]. The enzyme (CGTase) can be isolated from the culture broth of various bacilli. According to the nature of the later and the environmental conditions, three different CDs can be obtained in various proportions: α-,β-, and γ–CDs constituted, of six, seven, or eight, respectively glucopyranose units as described in Figure 4. Difference in steric requirements among the primary and secondary hydroxyls are such that CDs are cone-shaped, the narrower side bearing the primary hydroxyl groups and the other the secondary. This special conformation of the molecule results in external hydrophilicity and internal hydrophobicity.

The three natural CDs differ not only in their diameter, related to the number of glucopyranose units, but also in their water solubility [85]. The water solubility does not vary regularly from α- to β- to γ-CDs. In fact, the solubility at 25 °C is respectively 14.5, 1.85 and 23.2 g/100 ml. Thus, ß-CD, which for many years has been the most easily available and commonly employed, is the least water-soluble of the series. The

special conformation of CDs confers on them the ability to include inside their hydrophobic cavity various guest molecules on the condition that their size is compatible with the cavity of the host CDs. From the pharmaceutical standpoint, α-CD has a cavity (diameter ≈ 500 pm) too small to include most of the active molecules, β- and γ-CDs are more convenient (diameters ≈ 600 and 800 pm, respectively). The inclusion complex obtained exhibits new physicochemical properties, particularly those related to apparent water solubility and stability.

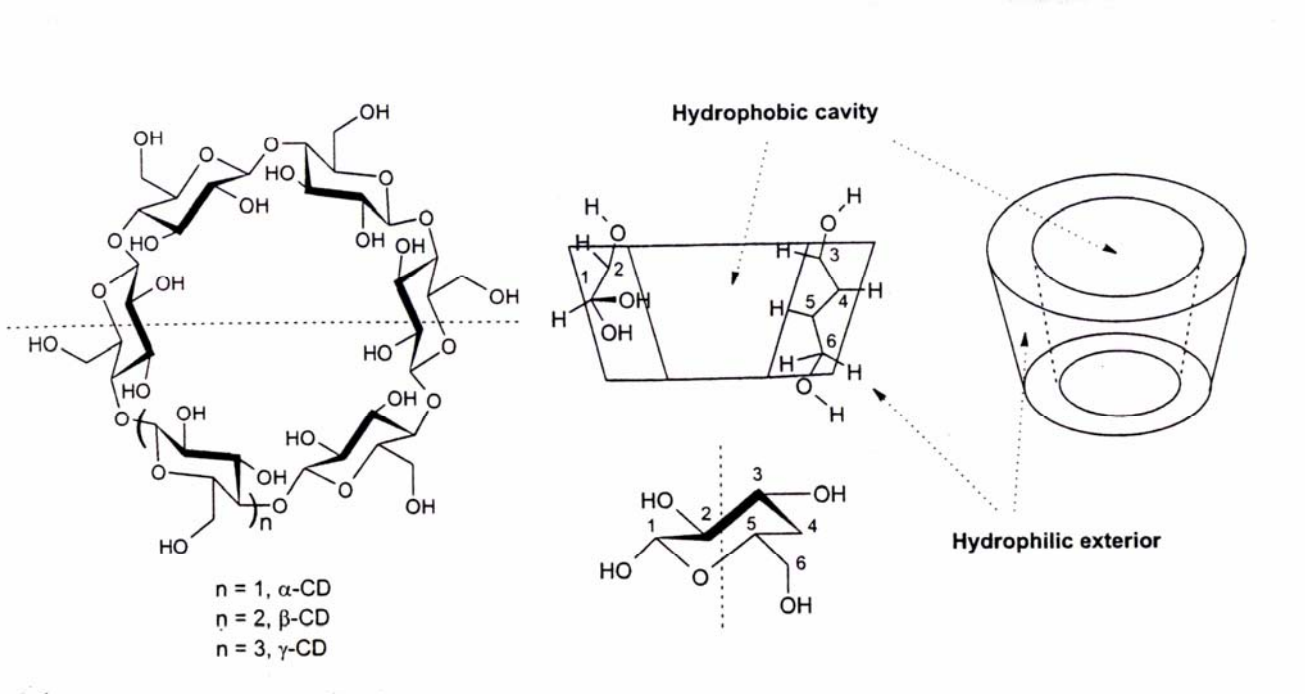


Figure 4: Structure of α-, β-, and γ -cyclodextrins

1.4.2. Properties

Because of the chemical structures and overall molecular shapes of α-, β- and γ-CDs, one of the most important characteristics of CDs is the formation of inclusion complexes with various guests. Guest compounds range from polar reagents such as acids and amines to highly apolar aliphatic and aromatic hydrocarbons and even rare gases. Recently, even some polymers have been found that can be included as guests in CDs [86]. CDs have some unique physical and chemical properties which are summarized in Table 5. The secondary OH-groups on the CD molecule (i.e. 2-OH and 3-OH on the glucosepyranose units) are somewhat more acidic than

the primary OH-group (i.e. 6-OH). Thus alkylation of 6-OH, the least sterically crowded functionalities, is favoured in strong basic solutions while alkylation of 2-OH, the most acidic of the hydroxyl groups but also the most hindered, is favoured in a weak basic solution [87]. Thus, some degree of regioselectivity is possible. CDs are stable in alkaline solutions. However, they are susceptible to acid hydrolysis. Partial acid hydrolysis of CDs produces glucose and a series of maltosaccharides [88]. The stability of CDs toward acid hydrolysis depends on the temperature and acidity where at pH > 3.5 and at a temperature < 60 °C, CDs are fairly stable.

	α-CD	**β-CD**	**γ-CD**
MW	972	1135	1297
Glucose units	6	7	8
Water solubility g/100ml	14.5	1.85	23.2
Cavity diameter (pm)	470-530	600-650	750-830
Total diameter (pm)	1460	1540	1750

Table 5: Some physicochemical properties of cyclodextrins

1.4.3. Toxicological considerations

Exhaustive tests on toxicity, mutagenicity and carcinogenicity of CDs and some of their derivatives have been carried out [89]. All toxicity studies have demonstrated that orally administered CDs are practically non-toxic, due to lack of absorption from the gastrointestinal tract [89]. Since November 13, 2000, ß-Cyclodextrin (ß-CD) is licensed in Germany as an additive in food [90]. ß-cyclodextrin was included as a food additive (E459) in the form of tablets and draggers with the limitation “ as much as necessary ”.

For use in textile finishing, ß-CD modified with a reactive group (monochlorotriazinyl group) is used. This anchor group reacts with the hydroxyl groups of cellulose and the cyclodextrin form permanent covalent bond [84]. Therefore also toxicological data for this cyclodextrin derivative are important. According to the OECD test this cyclodextrin derivative has no irritating or sensitising effects. Thus comparable results

for textile materials finished with this derivative are expected. These expectations are supported by the first clinical trials with T-shirts. No irritation of the human skin could be detected [91].

1.4.4. Cyclodextrin derivatives

Methyl cyclodextrins

Two types of methylated CDs are available: the dimethyl (on C2 and C6) and trimethyl (C2,C3 and C6) derivatives. These products are much more water soluble than the parent CD [92]. For example, at room temperature the water solubility of ß-CD increases from 1.85 g/100 ml to 57 and 31 g/100 ml, respectively, for the dimethyl and trimethyl derivatives. However, these products present a drawback: their dissolution is an exothermic phenomenon leading to a decrease in solubility when the temperature increases [93].

Hydroxypropyl cyclodextrins

Hydroxypropyl cyclodextrins which are a mixture of variously substituted CDs, are amorphous products and exhibit a very high water solubility: more than 50 g/100 ml for the 2-hydroxypropyl ß-CD, which is most often utilized [92,94].

Other derivatives

The two previous series of CD derivatives are those most commonly studied and employed and are readily available on the market. However, many other derivatives have been prepared and present high water solubility [92]. This is the case of the hydroxyethyl derivatives, which behave like and closely resemble the hydroxypropyl CDs [95], and the branched CDs (glucosyl and maltosyl derivatives) [96].

Sulfated and sulfoalkylated derivatives were described recently. They have a very high water solubility but are generally proposed for certain of their biological properties [97]. ß-CD polymers of low MW (2 to 5 CD units) are readily soluble in water, whereas those of high MW can only swell in water [98].

Carboxyethylmethyl cyclodextrins are derivatives with pH-dependent solubility [99]. The ß-CD derivative has a very poor solubility for low pH, increasing dramatically around pH 4.5 with a maximum for pH over 7.

Ethyl cyclodextrins are derivatives said to be water-insoluble but this characteristic seems to be debatable, probably as a function of the exact preparation method [100,101].

1.4.5. Ability to include guest molecules

The most important characteristic of CDs and their derivatives is their ability to include various or part of molecules inside their hydrophobic cavity, leading to an inclusion complex exhibiting new physicochemical and biological properties [102]. To form inclusion complexes, guest molecules must have a size and shape which will allow them to be at least partially accommodated in the CD cavity. For example, α-CD is too small to fully encapsulate a benzene ring but ß-CD can form inclusion complexes with benzene derivatives carrying bulky substituents such as *p-tert*-butylphenol [103]. The cavity of γ-CD is much wider and it can readily accommodate polycyclic aromatic hydrocarbons such as pyrene as well as steroids. Therefore, molecular size is a key factor determining the guest specificity of CD complexes. For the complex preparation several techniques are used to form cyclodextrin complexes; Co-precipitation, slurry complexation, paste complexation (kneading method), damp mixing and heating, extrusion and dry mixing [104].

Inclusion mechanism

The inclusion reaction can occur in solution, or at least in the presence of some humidity. The principle is the following: When a CD is dissolved in water, the water molecules inside the cavity are in a more hydrophobic environment and can not equilibrate their potentiality to create hydrogen bonds. Hence, from the thermodynamic standpoint, they are in a less stable condition. In such conditions, when a less hydrophilic molecule than water itself is in the CD environment, it is embedded into the cyclodextrin cavity. The supermolecule obtained can often be separated in solid form, either by spontaneous precipitation or after evaporation (or sublimation) of the aqueous phase. That means the main driving force of complex formation is the release of enthalpy-rich water molecules from the cavity. Inclusion of a guest molecule (or part of a guest molecule) in a CD is, in fact, a succession of equilibria:

Solid guest molecule ⇌ dissolved guest molecule,

Dissolved guest molecule + dissolved CD ⇌ dissolved inclusion complex,

Dissolved inclusion complex ⇌ solid inclusion complex.

In the equilibrium leading to the inclusion complex, this constant is the affinity constant (of the guest molecule for the CD host molecule) or stability constant (of the inclusion complex in the form of a supermolecule, not dissociated). A low value of this constant means that in solution the inclusion supermolecule will dissociate readily. On the contrary, a high value of this constant means that in solution the inclusion supermolecule will be predominant when compared with the dissociated form. Measurements of stability or equilibrium constants (K_c) or the dissociation constants (K_d) of the drug-cyclodextrin complexes are important since this is an index of changes in physicochemical properties of a compound upon inclusion. A wide variety of experimental methods have been employed for the measurement of the stability constants of CD-guest complexes [105]. These include microcalorimetry, electronic absorption (UV-vis), fluorescence, NMR and electron spin resonance (ESR) spectroscopy, pH potentiometry, kinetic experiments and solubility determination. Calorimetry is the only direct method for the measurement of CD-guest complexation enthalpy.

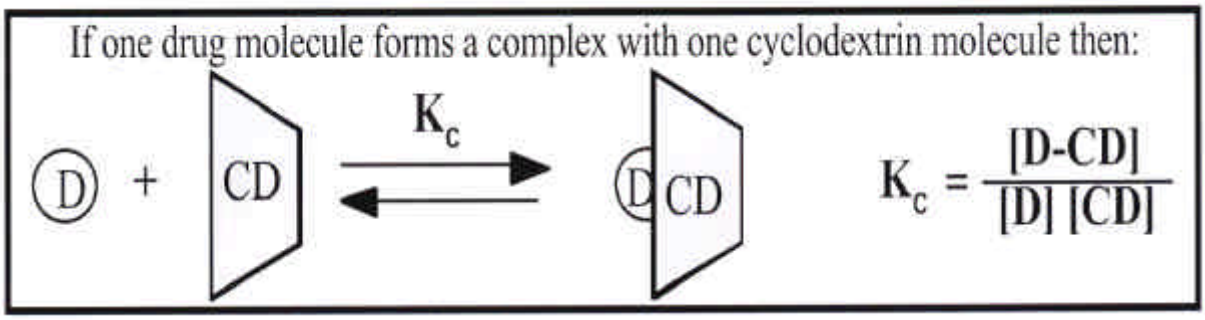


Figure 5: Schematic diagram showing the complexation process

Interest in inclusion complexes

Inclusion of an active ingredient molecule as a guest in a host CD molecule is a real molecular nanoscale encapsulation, and the resulting inclusion complex supermolecule has new physicochemical properties, stability and apparent solubility, resulting in new pharmacotechnical characteristics, formulation possibilities and consequently, better therapeutic efficacy. For example, ß-cyclodextrin forms a complex with nicotine. This property can be utilized in cigarette filters to remove most of the nicotine and tar from the smoke [106]. An odourless complex can also be prepared from onion and garlic oil. Allicin, one of the antibacterial but badly smelling components of garlic, forms stable complexes with cyclodextrins [106]. The explosive substance nitroglycerin also forms complexes with cyclodextrins. The crystalline complex is explosion-proof. Tablets prepared from that complex are more stable than tablets containing simply adsorbed nitroglycerin [106].

1.4.6. Applications of cyclodextrins

Pharmaceutical

One of the classic applications of CDs is in the field of pharmaceuticals. Pharmaceutical industries are always in need of new formulating aids, both for enhancing inadequate physical properties of new active ingredients and for reformulating existing drugs. CDs offer significant advantages over standard formulating aids. The complexes formed with the active ingredients stabilize, enhance their solubility, bioavailability and diminish their adverse effects. In short, CDs act as a drug delivery system and are potential drug delivery candidates in many applications because of their ability to alter the physical, chemical, and biological properties of guest molecules through the formation of inclusion complexes [107,108]. Their bioadaptability and multi-functional characteristics make them capable of alleviating the undesirable properties of drug molecules in various routes of administration including oral, rectal, nasal, ocular, transdermal and dermal [109-112]. The role of CDs in drug formulation and delivery is discussed in detail by Stella et al. [113].

Besides drugs, different peptides and proteins, oligosaccharides and oligonucleotides are also delivered by the formation of inclusion complex with CDs because of CDs capability of interacting with cellular membranes and giving rise to improved cellular uptake [109,114,115].

Environmental

Cyclodextrins (CDs) can play a major role in environmental science in terms of solubilisation of organic contaminants, enrichment and removal of organic pollutants and heavy metal complexes from soil, water and atmosphere [116]. CDs are also applied in water treatment to increase the stabilizing action, encapsulation and adsorption of contaminants [117]. Using CDs, highly toxic substances can be removed from industrial effluent by inclusion complex formation. In the mother liquor of the insecticide trichlorfon, the uncrystallizable trichlorfon can be converted into a ß-CD complex and in a single treatment 90 % of the toxic material is removed [118]. Wastewaters containing environmentally unacceptable aromatic compounds such as phenol, p-chlorophenol and benzene after treating with ß-CD have considerably reduced levels of these aromatic hydrocarbons from their initial levels [119]. CDs are used to scrub gaseous effluent from organic chemical industries [118]. CD complexation also resulted in the increase of water solubility of three benzimidazole-type fungicides (thiabendazole, carbendazim, and fuberidazole) making them more available to soil [120]. In addition to their ability to increase the solubility of the hydrocarbon for biodegradation and bioremediation, CDs also decrease the toxicity resulting in an increase in microbial and plant growth. ß-CD accelerated the degradation of all types of hydrocarbons influencing the growth kinetics, producing higher biomass yield and better utilization of hydrocarbon as a carbon and energy source. The low cost, biocompatible and effective degradation makes ß-CD a useful tool for bioremediation [121]. Another important role of CDs in environmental protection is its usage in insecticide formulation [122].

Textile industry and packing

Textile finishing is another area in which CDs are increasingly attracting attention. Fabrics can be imbued with chemicals by means of CDs to give them novel properties. In order to permanently transfer the versatile properties of CDs to textiles, DTNW (Deutsches Textilforschungszentrum Nord-West e.V., Krefeld) researchers covalently attached reactive CD derivative with monochlorotriazinyl (MCT) substituents to the fibre [123,124]. In Table 6, different possibilities of fixing CDs onto various fibre polymers are summarized. This cyclodextrin acts like a reactive dye and can be fixed onto the surface of cotton fibre by employing a conventional dyeing process [125]. Owing to the fact that the monochlorotriazinyl derivative of ß-CD does not possess any allergic potential, the modified fabrics may come into contact with human skin without causing any harm [126].

Fixation Mode \ Fibre Material	Cellulose	Wool	Polyamide Fibre	Polyester Fibre	Polyacrylonitrile Fibre
Cross-linking agents	+	–	–	–	–
Ionic interactions	–	+	+	–	+
Covalent bonds	+	+	+	–	–
Van der Waals interactions	–	–	+	+	+

Table 6: Strategies for the fixation of CDs onto the surface of different fibres [106]

This substituted CD provided excellent textile finishing to cottons, blended materials and woollens. After the fixation of CDs onto the fibre surface, the characteristic behaviour of some fibre materials changes. Thus, for example, the hydrophobic character of polyester fibres disappears [127]. The fixed CDs are able to form inclusion compounds as the molecules in solution. Thus, sweat components can be incorporated into the cyclodextrin cavities of a treated fabric, and the release of odour is reduced. This; Industrially used for a few years. On the other hand, it is possible to complex perfumes with CDs [128]. In the presence of moisture, these complexed perfumes are released. Curtains made from textiles with fixed CDs are also able to complex nicotine, tar, and

other molecules from the air. During a normal washing process, these complexed molecules can be removed. Using hydrophobic tosyl derivative of ß-CDs, 3-fold increase in the binding of fluorescent dye to the polyester fibre was attained [129]. A lot of research studies have been carried out in DTNW concerning cyclodextrins and its application in textile finishing. CDs were used by DTNW in dyeing process as auxiliaries. In the high temperature dyeing of polyester fibre (PET), CDs can also act as levelling agents [130]. However, if the stability of the complex with the dye molecule is small, which is the case with large dyestuff molecules, no effect upon levelness is observed. Hence the use of CDs as levelling agents is limited. The trichromic dyeing of cotton with direct dyestuffs is, however, influenced by the presence of CDs [131].

Dyeing processes with solid cyclodextrin-dye complexes were also carried out by DTNW researchers. The preparation of solid dye complexes with CDs is easily accomplished [106]. Solid complexes of ß-CD with direct, reactive, acid, and disperse dyes can be prepared. All these complexes can be successfully used for the dyeing of different fibres [132]. The main advantage is that no further auxiliaries have to be added to the dye bath. The use of cyclodextrin complexes with disperse dyestuffs for PET-dyeing results in a very high bath exhaustion, whereas in a standard disperse dyeing process, a considerable number of the dye molecules remain unfixed in the bath. The amounts of the remaining disperse dyestuffs in the waste water are significantly reduced when cyclodextrin-dye complexes are used in the dyeing process instead of conventional dyestuff formulations [132]. Thus the industrial use of cyclodextrin-dye complexes will be favoured for both ecological and economical reasons. In washing process, if CDs are present in textile washing process, they are able to remove most of the adsorbed surfactants which are normally used during any textile-processing step [133]. In absence of CDs, these adsorbed surfactants affect the dyeability, the wettability and the absorptivity of the textile surfaces. Furthermore, the presence of adsorbed surfactants on the surface of a fibre also reduces the quality of hydrophobic finishing. In addition, the adhesive strengths of coatings on the textile may be reduced, which is, for example, important for the production of air-bags used by the automobile industry [106].

CDs also play a major role in the packing industry. CD inclusion complex containing oily antimicrobial and volatile agents are coated on a water-absorbing sheet with a natural resin binder, which is used for wrapping fresh products [134]. It was found that food-packaging bag manufactured using CD with ethylene-tetracyclo-3-

dodecane copolymer, showed no odour and good antifungal properties after 1 week of storage at room temperature, which proved useful for food-packaging materials [135].

Cosmetics, toiletries and personal care

Cosmetic preparation is another area which demands a lot of CD use mainly in volatility suppression of perfumes, room fresheners and detergents by controlled release of fragrances from inclusion compounds. The interaction of the guest with CDs produces a higher energy barrier to overcome volatilization, thus producing long-lasting fragrances [136]. The major benefits of CDs in this sector are stabilization, odour control, process improvement upon conversion of a liquid ingredient to a solid form, flavour protection and flavour delivery in lipsticks, water solubility and enhanced thermal stability of oils [137]. Some of the other applications include use in toothpaste, skin creams, liquid and solid fabric softeners, paper towels, underarm shields [109].

The use of CD-complexed fragrances in skin preparations such as talcum powder stabilizes the fragrance against the loss by evaporation and oxidation over a long period. The antimicrobial efficacy of the product is also improved [129]. Fragrance is enclosed with CD and resulting inclusion compound is complexed with calcium phosphate to stabilize the fragrance in manufacturing bathing preparations [138]. CD-based compositions are also used in various cosmetic products to reduce body odours [139]. Dry CD powders of size less than 12 µm are used for odour control in diapers, menstrual products, paper towels, etc., and are also recommended in hair care preparations for the reduction of volatility of odorous mercaptans. The hydoxypropyl ß-CD, either alone or in combination with other ingredients, provides improved antimicrobial activity [140]. Dishwashing and laundry detergent compositions with CDs can mask odours in washed items [141].

Solubility improvement

The guest molecule is generally a poorly water-soluble active ingredient included in a water-soluble CD. The resulting inclusion complex benefits from the hydrophilic external part of the CD and has a higher solubility than the free active ingredient. In fact, the solubility observed is not that of the active ingredient, but of the inclusion complex itself, and thus there is no real increase in solubility of the active ingredient, but an increase in its apparent solubility. What is taken into account in the solution is the whole: Dissolved active ingredient and dissolved inclusion complex.

It can happen that the increase in apparent solubility is only momentary. This is the case when the stability constant of the inclusion complex is low, because it can dissociate rapidly, and the free insoluble active ingredient can reprecipitate from the medium. On the contrary, if the stability constant is high, then the improvement in stability can be prolonged.

Bioavailability improvement

In the case of active ingredients exhibiting a poor bioavailability due to water insolubility or low solubility, but without absorption problems, the improvement in apparent solubility can improve the bioavailability [142].

The mechanism involved is as follows [143]. In the presence of biological fluids, the solid inclusion complex dissolves and dissociate more or less according to its stability constant. The three components existing in equilibrium are in contact with the biological membrane: inclusion complex, empty CD and free active ingredient. Both the inclusion complex and the CD are too hydrophilic at their external part to be absorbed significantly by the lipidic membrane. On the other hand, the poorly water-soluble active ingredient, after its release from the inclusion complex, is available for absorption through the membrane.

Some remarks concerning this general mechanism must be made. It is necessary to have good water solubility of the inclusion complex; otherwise its dissolution is so slow before dissociation that the release of the active ingredient is very slow, and the bioavailability may not be improved at all. The stability constant of the inclusion complex must not be too high, because it is the very slow dissociation which is the limiting factor, and here, too, the bioavailability may not be improved. If the active ingredient is highly water-insoluble, the stability constant must not be too low, otherwise the large amount of released active ingredient my reprecipitate in the biological fluids before absorption.

In this thesis the antimycotic influence of ß-CD complexes will be measured and evaluated by the so called laser nephelometry in microtiter plates. This method is new and rapid in comparison with other traditional turbidimetric ones. In addition, the solubility of the prepared inclusion complexes will be measured and evaluated by the same method. This part of the thesis will be discussed in details later in chapter 3.

Chapter 2

Experimental part

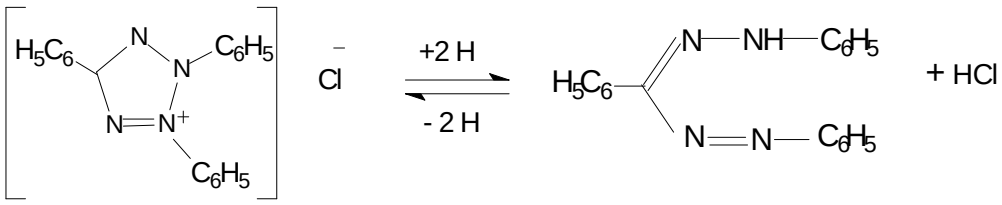
This chapter divided into three sections, the first and the second section discuss in details all experimental work related to chitosan and its application in textile and dentistry. The third and the last section discuss in details the experimental work of ß-cyclodextrin as a drug delivery system and its pharmaceutical applications. Before starting the experimental part, we will talk about the interesting new methods used in this thesis.

2.1. Background of methods

2.1.1. Tetrazolium/formazan-test method (TTC)

The TTC-test method is used in this thesis to measure the antibacterial efficiency of immobilized chitosan fixed either on cotton fabrics or as film coated on Molloplast® B-soft liner denture based materials.

Tetrazolium salts as TTC and formazans have been known in chemistry for about a hundred years. Besides their role in chemistry and industrial technology, tetrazolium salts and formazans are now widely applied in different branches of the biological science e.g. in medicine, pharmacology, immunology and botany, but especially in biochemistry and histochemistry. The tetrazolium/formazan couple is a special redox system acting as proton acceptor or as oxidant as described below in Figure 6 [144]. In the presence of bacteria, TTC is reduced to red formazan. The red formazan obtained indicates the activity and viability of the cells. The absorbance of formazan, measured at 480 nm, is directly proportional to the viable active cells. Therefore, the TTC-test method is considered a fast method for evaluating the antibacterial activity of cotton fabrics finished with chitosan.



2,3,5-triphenyl-2*H*-tetrazol-3-ium 1-phenyl-2-[(*Z*)-phenyl(phenylhydrazono)methyl]diazene

(TTC; colourless) (Formazan; deep red)

Figure 6: Mechanism of the tetrazolium/formazan system

2.1.2. Laser nephelometry in 96-microtiter plate

Laser nephelometry has been shown to be a reliable technique for the measurement of not only the drug solubility in 96-well plate format but also the anti-mycotic influence of the drug, whether alone or in complex with cyclodextrins.

The laser nephelometer used in this thesis is the NEPHELOstar Galaxy® (BMG LABTECH GmbH, Germany) described in Figure 7. This instrument is based on a laser nephelometer employing a polarized helium-neon laser that emits in the red at 632.8nm. The laser beam is passed through the well in a vertical and concentric path. If the laser beam is passed through an empty well or a clear liquid within the well, then the light beam is not scattered and passed through unchanged. In turbid suspensions (Fungi in this study), scattered light is detected by a photo detector at right angles to the incident laser beam. The NEPHELOstar will only measure the light that is forward scattered as described in Figure 7. The energy of the scattered light is directly proportional to the particle concentration in the suspension for up to 3 orders of magnitude [145]. This method is novel, rapid and simple in comparison with other traditional turbidimetric methods. Nephelometry has the advantage that it detects a drug that has been separated out of solution.

Figure 7: a) Laser nephelometry instrument

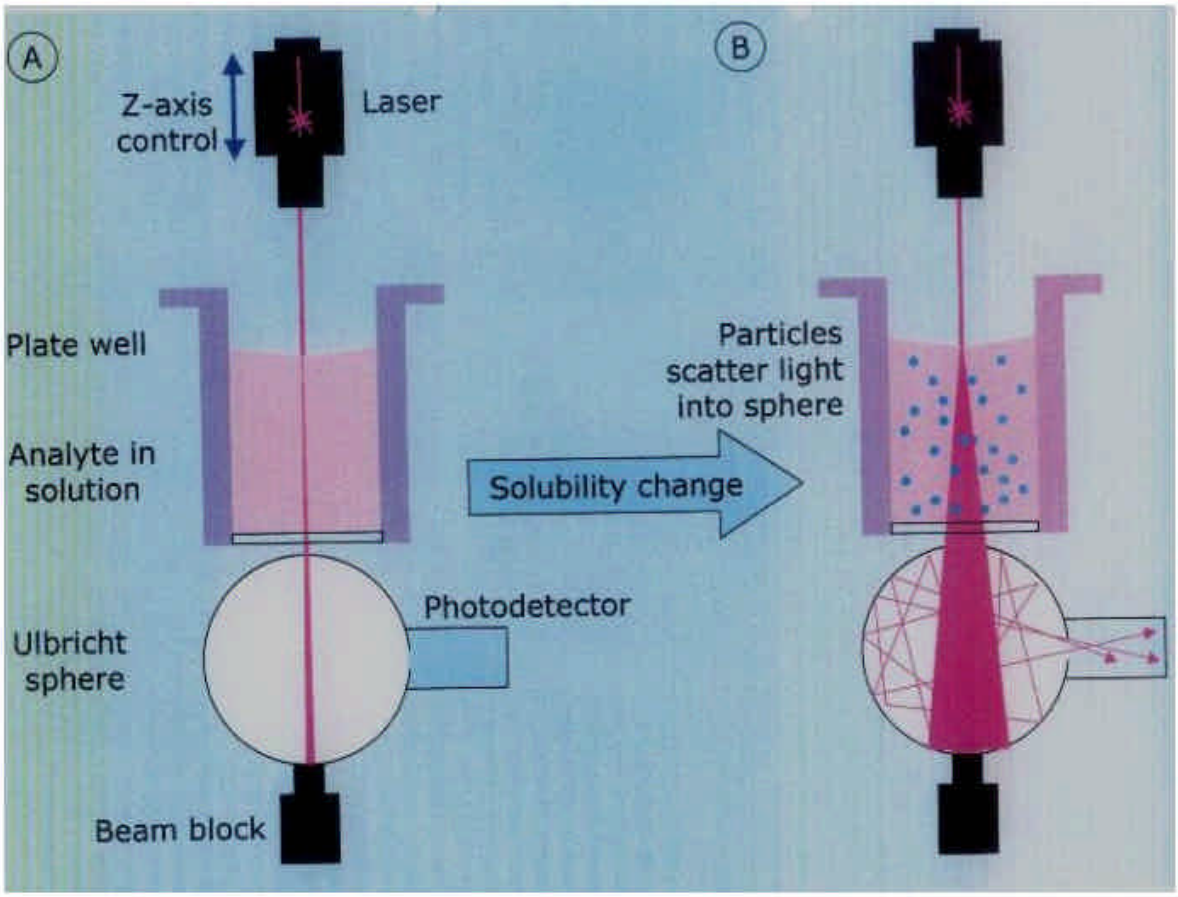


Figure 7: b) Principle of Laser nephelometry

2.1.3. Polyelectrolyte titration method

The electric charge of colloidal systems like gelatine or biopolymer solutions (chitosan in this study) is of great importance for their stability in respect of flocculation. Electrokinetic phenomena like zeta potential and charge density are widely used to characterize and predict the stability of these systems. With different experimental setups one can either determine the velocity of particle movement or mass transport (electrophoresis, electroosmosis) by applying an external electric field or on the other hand induce an electrical potential (streaming potential) by applying an external pressure. The amount of ionic charges on the particle surface can be measured only by a few methods.

In this work the amount of charged groups of chitosan add on cotton is measured by titration with a counter-charged polyelectrolyte solution with well known content of charged groups. The titration is monitored by a new streaming potential instrument which detects the sign of the charged groups and shows a streaming potential of the colloidal suspension in arbitrary units. The main element of the particle charge detector (PCD) is a cylindrical vessel (1) with an oscillating displacement piston (2) forcing the colloidal solution through the annular space between piston and bore [146]. The movement of the piston creates a streaming potential between two electrodes (3) above and below the bore as described in Figure 8.

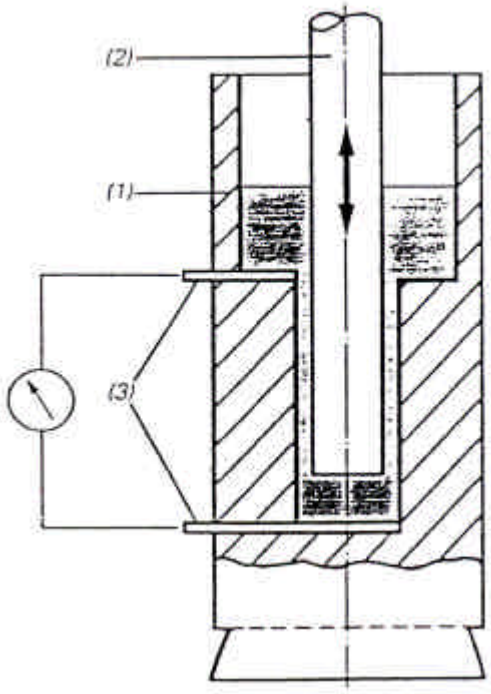
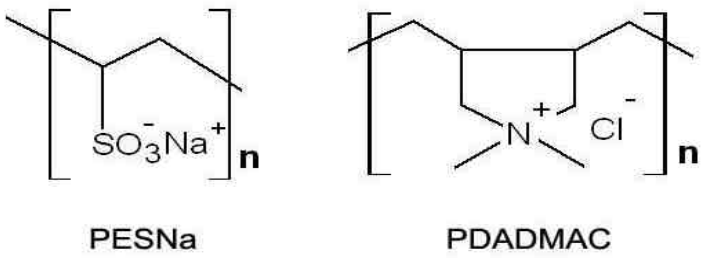


Figure 8: Particle charge detector (PCD 03 PH)

The adsorption of polyelectrolyte to the surface of the vessel and piston is effected by Van der Waals forces. The flow forced by the oscillating piston causes the diffuse outer part of the electric double layer to be sheared off. The induced streaming potential is processed electronically and monitored on a display.

Polyelectrolyte titrations are performed with cationic charged solutions of poly-di-allyl-di-methyl-ammonium-chloride (PDADMAC) in case of anionic systems and with anionic poly-ethylensulfonic acid, sodium salt (PESNa) in case of cationic systems. The concentration used is 0.001 N. The equivalence of (PDADMAC) is controlled by titration with (PESNa), which contains one charge per monomer unit.



PESNa PDADMAC

Figure 9: Unit structure of cationic and anionic polyelectrolyte

2.2. Chitosan in textile applications

2.2.1. Instrumentation analysis

Gel permeation chromatography (GPC); a laser light scattering device PN-3000 (15° and 90°) together with a refractive index detector PN-1000, were from Post-nova analytics, Eresing, Germany. Columns Nucleogel GFC 1000-8, (Macherey-Nagel GmbH & Co. KG, Düren, Germany) as well as Gral 3000 by PSS (Polymer Standards Service GmbH, Mainz, Germany).

The FTIR instrument was from Biorad, Model FTS-45, Germany.

The ^{13}C-n.m.r-CP-MAS-spectra were carried out at the University of Duisburg-Essen using Fa. Bruker ASX 400 (^{1}H-frequency = 400 MHz, ^{13}C-frequency = 100, 6 MHz) and MAS-Probe, 7mm Rotors was from Analytik GmbH, Rheinstetten, Germany.

The Kjeldahl apparatus; model Gerhardt Vapodest 1, was from Germany.

Datacolor type international 600 018 was from datacolor AG, Switzerland.

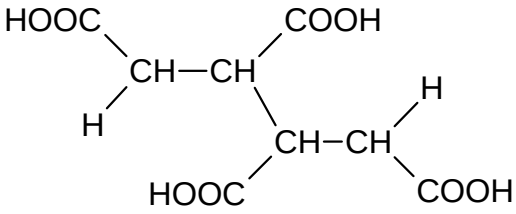
Zwick, textile material testing, was from England.

Polyelectrolyte titration system, Particle charge detector (PCD 03 PH), was from Mütek Analytic GmbH, Herrsching, Germany.

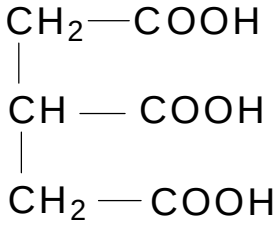
2.2.2. Chemicals and materials

Chitosan samples of different molecular weights (coded as: He 75-100, He 80-1000, He 80-4000, He 85-15, He S 85-60, He 85-250 and He 85-500) were kindly supplied by Heppe GmbH, Queis, Germany. The first number in case of Heppe products represents the degree of deacetylation (DD) used as received from the company while the second number represents the Brookfield viscosity. Other samples coded as Bil 02, Bil 03, Bil 04 and Bil 05 were obtained from FTZ, Bremerhaven, GmbH, Germany. Bilb 99 and chitopure 21 were obtained from Marine Bio Products GmbH, Germany. Cyanuric chloride was from Fluka, Germany. Propane-1,2,3-tricarboxylic acid (PTCA), sodium acetate trihydrate, butane 1,2,3,4 tetracarboxylic acid (BTCA), monochloroacetic acid, Standard I-nutrient broth medium (SI), Standard I- nutrient agar (SI-agar) and 2,3,5- triphenyltetrazolium chloride (TTC) were supplied by Merck, Germany. 2,3- epoxy propyltrimethyl ammonium chloride (Quab® 151) was kindly supplied by Degussa AG, Germany. 2,4-dichloro-6-methoxy-s-triazine was synthesized according to [147]. Two types of dyestuff were used, acid dye namely, (Alizarin

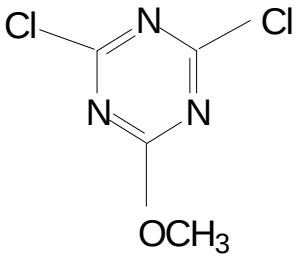
Red S Monohydrate, C.I. 58005), and reactive dye namely, Cibacron, Tuerkis, 2G-E, Ciba-Geigy. All other chemicals and solvent are of laboratory grade.



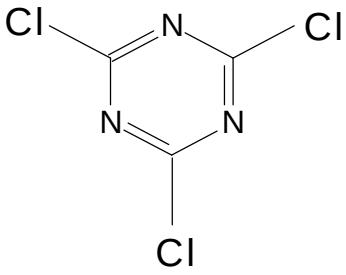
a) Butantetracarboxylic acid



b) Propane tricarboxylic acid



c) 2,4-Dichloro-6-methoxy-s-triazine



d) Cyanuric chloride

Figure 10: Chemical structures of different types of anchors

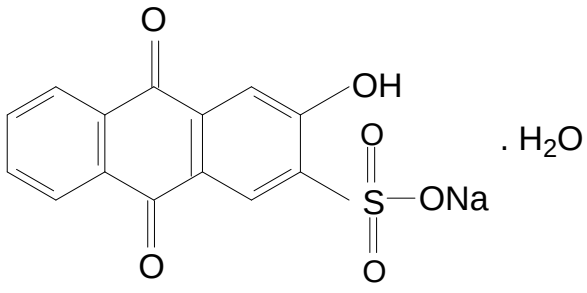


Figure 11: Chemical structure of Alizarin Red S Monohydrate

2.2.3. Test fabrics

As a test fabric, 100 % cotton (Style S/400, 102 g/m^2, pleine weave) which was bleached and desized was purchased from TESTEX, Germany. Prior to application, the fabric was further purified by washing with warm water and methanol. For the washing, 1 g/l of non-ionic detergent (Marlipal® O 80/13); kindly supplied by Degussa AG, Germany was added. After washing, the fabric was rinsed with warm water three times and dried, climatized for 24 h (22 °C, 65 % rel. humidity) and weighted before using.

2.2.4. Preparation of micro organisms

Escherichia coli (*E.coli*) DSMZ 498 and *Micrococcus luteus* ATCC 9341 were used as examples of gram-negative and gram-positive bacteria. One well-isolated colony was transferred aseptically, using a wire loop, to a 50 ml conical flask containing 25 ml SI-medium. The flask was incubated at 37 °C for 24 h and then the grown bacteria were diluted with sterile saline to a final working concentration.

2.2.5. Characterization of chitosan

Molecular weight (MW)

The determination of the average molecular weight for either chitin or chitosan posses a number of problems. Chief among these in the case of chitosan are the possible presence of microgels due to uneven treatment during the deacetylation step, and the tendency of the polymer chains to aggregate on standing in solution [148]. Although a number of techniques have been applied to the problem only light scattering and gel permeation chromatography (GPC) have been used to any extent. GPC is a frequently used technique for determination the molecular weights and molecular weight distributions (MWD) of polymers, and it is not surprising that it has been employed in studies on chitosan [149]. The cationic nature of chitosan in acid solution causes difficulties in using GPC for molecular weight determinations of chitosan. For the separation process to operate correctly it is necessary that there is no interaction between the gel and the polymeric solute, so in the case of polyelectrolytes any electrostatic effects, whether of repulsion or attraction, must be eliminated. Conventional

GPC gels carry a negative surface charge so that the protonated chitosan chain would be expected to be strongly adsorbed onto the gel through electrostatic attraction.

In this thesis GPC measurements of chitosan solutions were performed using commercially available columns Nucleogel GFC 1000 - 8, as well as Gral 3000. For calibration standards of pullulan (non charged polysaccharide molecule) of MW 100, 200 and 500 kDa were used. Aqueous buffer solution (acetic acid/sodium acetate 0.5/0.3 M/l) as a mobile phase with flow rate of 1 ml/min was used. Pullulan is a homopolysaccharide that is elaborated by many species of the fungus *Aureobasidium*, specifically A. pullulan. Pullulan is a highly water soluble linear polysaccharide of D-glucopyranose residues, containing a-1-4 and a-1-6 glycosidic linkages in the ratio of 2:1 [150]. Pullulan readily dissolves in water to give stable and viscous solutions that do not gel. The average molecular weight (MW) of chitosan samples determined by GPC instrument are listed in Table 7.

Chitosan codes	**Average MW . 10^{-5} Da**
He 75-100	1.53
He 80-1000	4.16
He 80-4000	3.45
He 85-15	1.28
He S 85-60	1.36
He 85-250	2.70
He 85-500	3.01
Bil 02	1.90
Bil 03	2.06
Bil 04	3.40
Bil 05	0.82
Chitopure 21	0.65
Bil 99	0.40

Table 7: Average molecular weight of chitosans measured by GPC

Degree of deacetylation (DD)

To determine the DD of chitosan samples as example Bil 99, potentiometric titration method proposed by Koetz and Kosmella was employed after slight modification [151]. 50 mg of chitosan was dissolved in 3 ml of freshly prepared 0.1 M HCl. To the excess component (chitosan hydrochloride) a defined solution of an opposite charged titrant component was slowly added under stirring at a flow rate of 1.2 ml/min to maintain equilibrium condition. The changes in turbidity, conductance, and the pH were automatically registered as functions of the titrant volume described in Figure 12. The potentiometric back titration of chitosan hydrochloride solution is given together with the turbidimetric titration curve. The first equivalence point of the pH-curve directly corresponds to the degree of dissociation (a = 0). The second equivalence point (a = 1) is in good agreement with the flocculation point; that is the maximum of the turbidity curve. From the NaOH consumption between (a = 0) and (a = 1) the degree of deacetylation is directly determined to be 85 %.

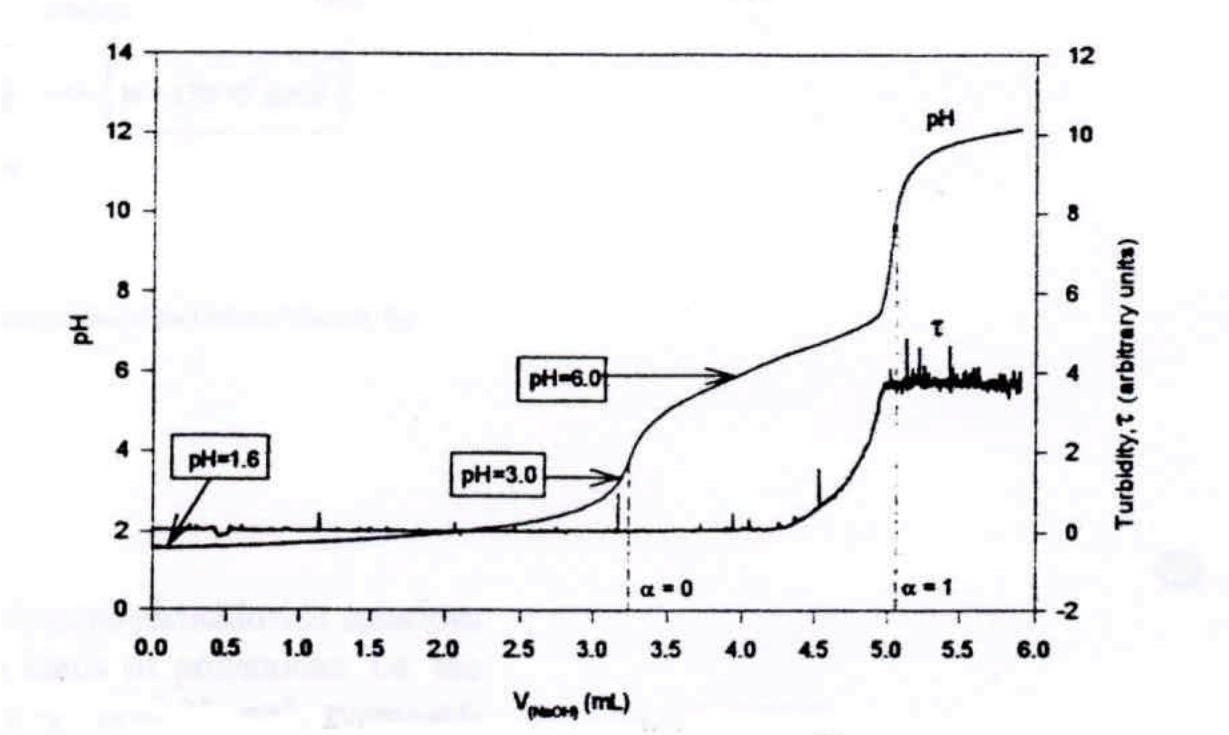


Figure 12: Back titration of chitosan hydrochloride

2.2.6. Fabric treatment with chitosan

For the crosslinking of chitosan to cotton fabrics different anchors were used: cyanuric chloride (CNC), (2,4-dichloro-6-methoxy-1,3,5-triazine) (MCNC), (propane 1,2,3-tricarboxylic acid) (PTCA) and (butane 1,2,3,4-tetracarboxylic acid) (BTCA) (Figure 10). The concentration range of each anchor was from 5 and 8 x 10^{-4} mol/g fibre. At first CNC and MCNC were dissolved in dioxan, then the cotton fabrics were immersed in a solution containing the dissolved anchors and chitosan solution. But in case of carboxylic anchor groups, the fabrics were immersed in an aqueous solution containing the crosslinking agent. In addition, 0.6 mole of sodium acetate trihydrate (catalyst) to 1 mole anchor was used. The fabrics were padded in this solution to a 100 % wet pick-up (WPU). Chitosan coded as (He 85-60) was dissolved in an acetic acid solution (mass concentration of 1 %). Chitosan was applied to cotton fabrics by a pad-dry-cure method. Cotton fabrics were padded twice with the chitosan solution (1 %) to a 100 % WPU. The padded fabrics were then dried at 80 °C for 5 minutes and cured at 90-170 °C for 3 minutes depending on the anchor group types. The finished fabrics were washed with water, followed by treatment in aqueous solution of acetic acid 1 %. Finally the fabrics were washed in cold water to neutral and dried at ambient temperature. Figure 13 shows the reaction mechanism.

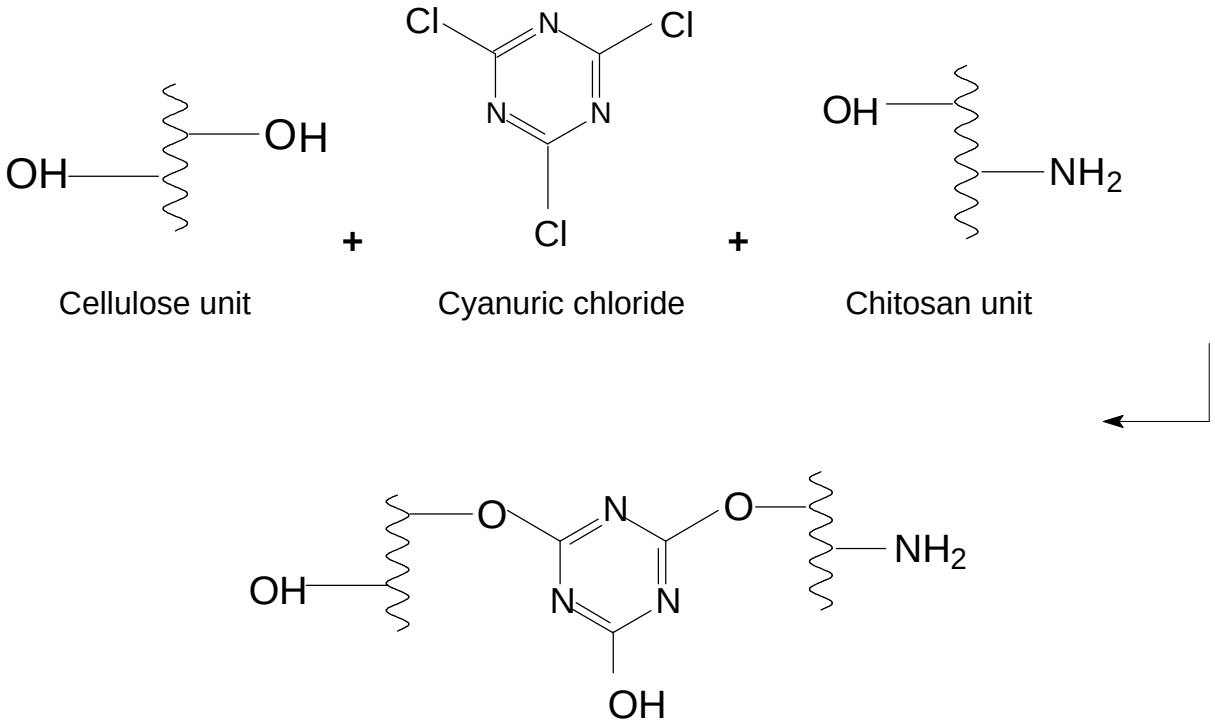


Figure 13: Assumed structure of chitosan-finished cotton using CNC anchor group (Cl - lost by hydrolysis)

2.2.7. Polyelectrolyte titration of treated cotton fabrics

Polyelectrolyte titration was carried out in order to determine the amount of charged chitosan groups added on cotton. The following is the procedure:

1 g climatized treated cotton fabrics were immersed in 100 ml acetate buffer solution (pH 4.7). To this solution 100 µl Marlipal was added. The cotton samples were left with shaking for 2-3 h. Then the cotton fabrics were washed several times with distilled water and dried at ambient conditions. 50 ml anionic polyelectrolyte (0.001 N polyethlenesulfonic acid sodium salt; PES-Na) (Figure 9) and 100 µl Marlipal were added with stirring for 3 h to the cotton fabrics. After stirring, the solutions with the cotton fabrics were filtered out. 10 ml from the filtrate was titrated against cationic polyelectrolyte (PDADMAC 0.001 N) (Figure 9). The volume consumed during the titration was called (V_1). For calibration, 10 ml (PESNa) was titrated against (PDADMAC), the volume determined was called (V_2). The amount of titer consumption at the end point of titration can be used for the calculation of accessible charges according to the following equation:

milliVolt. equivalent (mV.eq.) = $[(V_2-V_1)x\ 0.001] /0.2$.

i.e., V_1 = Consumption of (PDADMAC) which is equivalent to the unreacted (PESNa) (eq/ml),

V_2 = Consumption of (PDADMAC) equivalent to 10 ml (PESNa) (eq/ml), for the correction of the volume which equivalent to the 1 g cotton sample, we multiply the (V_2-V_1) by factor 5.

2.2.8. Synthesis of carboxymethyl chitosan (CMCTS)

The experimental technique adopted for carboxymethylation of chitosan was as follows: a solution of 32 g of sodium hydroxide in 20 ml of water was mixed with 16 g of chitosan suspended in 100 ml isopropanol. The mixture was left under stirring for 30 min at room temperature. To this mixture 34 g of monochloroacetic acid was added in portions and the total content of this mixture was was subjected to continuous stirring at 60 °C for 3 h. The mixture was neutralized using glacial acetic acid and the chitosan were precipitated by adding acetone. Finally, the modified chitosan was carefully washed successively by isopropyl alcohol several times and finally dried at -60°C [152]. The final product is soluble in water.

2.2.9. Cationization of cotton fabrics

Cationization of cotton fabrics were carried out using pad-dry-cure method [153]. The experimental conditions were adopted as follows: The cotton fabrics were padded in solution containing Quab® 151 (50,85,100 and 140 g/l) and NaOH (1:1 molar ratio), acetone was added as proper solvent for the reaction medium then, the fabrics were squeezed between two nips and dips to a 100 % WPU. The cotton fabrics were dried at 80 ^{o}C for 3 min, then cured at 120 °C for 5 min.

2.2.10. Reaction of CMCTS with pre-cationized cotton

Reaction of CMCTS with pre-cationized cotton was carried out using cold pad-batch method. Pre-cationized cotton with Quab® 151 is allowed to react with CMCTS under the following experimental conditions: The cotton fabrics were padded in two dips and two nips in an aqueous solution containing CMCTS (20-50 g/l). The treated fabrics were then squeezed to 100 % WPU. The finished fabrics were kept in plastic bags at 60 °C overnight. At this end the finished fabrics were washed several times with water and finally dried at ambient laboratory conditions.

2.2.11. Dyeing of finished cotton fabrics

Dyeing of pre-cationized cotton fabrics and cationized cotton finished with CMCTS were carried out without addition of salt using acid dye, namely Alizarin Red S monohydrate, and reactive dye, namely Cibacron. The dyeing bath concentration was 500 mg/l and the material to liquor ratio was 1:50. The aqueous solution of the dye containing the samples was put in shaking water bath at 80 °C for 1 h. At this end of dyeing, the samples were washed several times with boiling water and 0.1 % Marlipal, and finally washed with cold water and dried at ambient conditions.

2.2.12. Textile testing and analysis

Nitrogen content of the cationized and CMCTS-treated samples was determined according to the Kjeldahl method [154].

Crease recovery angles (CRA) of the treated and untreated samples were measured according to DIN 53 890 standard test method [155].

Tensile strength (TS) and elongation at break (E %) were measured according to a standard test method [156].
Colour measurements as K/S were carried out using datacolor type international [157].
The wettability test according to TEGAWA was applied [158].
Both chitosan and chitosan derivative CMCTS were analysed and characterized by FTIR and solid state ^{13}C- n.m.r. spectroscopic analysis respectively.
Antimicrobial activity assays were carried out according to the method described in ref. [55]. Details about this method and another methods used are described in the following section.

2.2.13. Antimicrobial activity measurement of chitosan

Quantitative suspension method

The quantitative suspension method was used to measure and evaluate the antimicrobial activity of chitosan in solution. Stock solutions of chitosan were prepared (1 % in acetic acid). Using sterilized tubes, two fold serial dilution of chitosan was done using acetic acid 1 %. Before sterilizing, the pH of the medium was adjusted to 6.0 with 0.1 N NaOH. All tubes were inoculated with cells of *E.coli* (10^4 Cfu/ml) at 37°C. After certain period of times (1, 2 and 24 h) surviving cells were counted by spreading on nutrient agar plates (0.01 ml diluted bacteria suspension; 9 cm diameter of plate). The counting was done in triplicate each time [159]. The initial cell concentration was determined by a similar spread plate method.

TTC-test method

To do this test circular swatches of test fabrics finished with chitosan, its derivative and control cotton fabric were cut into a small size of a diameter (3.8 ± 0.1) cm. The number of swatches to be used was 6. Both of the test and control swatches were sterilized at 110 °C before incubation. All swatches were stacked in 40 ml SI-medium containing 10 µl of (10^8 Cfu/ml) challenge micro organism as an inoculum volume, then all flasks were incubated with shaking at 37 °C/200 rpm for 3–4 h, then 1 ml from each flask containing the test fabrics finished with chitosan and the control was added to sterilized test tubes containing 100 µl TTC (0.5 % w/v). All tubes were incubated at 37 °C for 20 min. The resulted formazan was centrifuged at 4000 rpm/3 min followed by decantation of the supernatants. The pellets obtained were resus-

pended and centifugated again in ethanol. The red formazan solution obtained at the end which indicated the activity and viability of the cells was measured photometrically at 480 nm.

2.3. Chitosan in dentistry

In this section chitosan films will be applied, as antimicrobial coating agent, on Molloplast® B-soft liner denture based material in order to develop potentially biocompatible soft denture liners coated with chitosan that would not need to be removed and of course can deliver medication to treat the so-called denture stomatitis. Molloplast® B-soft liner is a synthetic polymer with viscoelastic property made from poly ethyl methacrylate polymer and n-butyl ester monomer.

2.3.1. Chemicals and materials

Artificial saliva was prepared according to Öko-Tex Standard 201 M-9-A, c/o TESTEX, Zürich. Gypsum mold, dental wax pattern, dental flask, and heat cured soft liner Molloplast® B, were from DETAX, GmbH & CO.KG, Ettingen, Germany.

2.3.2. Test organism

In this study *Escherichia coli* (*E.coli*) DSMZ 498 and *Micrococcus luteus* ATCC 9341 were used as non pathogenic substitutes for gram-negative and gram-positive bacteria. Details of preparation are described as before in section 2.2.4.

2.3.3. Molloplast â B sample preparation

In this study heat cured soft liner was prepared as described in Figure 14. At first the dental wax pattern was cut using a wax knife in circular disk with a definite diameter and thickness. Using a dental flask, gypsum was mixed with water and poured to the lower flask compartment until the top. The prepared wax pattern disk samples were embedded in the superfacial gypsum mold surface. After final setting of the gypsum, the upper flask compartment was added and soft gypsum was added in excess. The flask was bench pressed until final plaster setting occur. After plaster set up, it was boiled for 10 min, then the two flask compartments were separated and the remnant sample waxes were washed by running boiling water. After bench cooling of

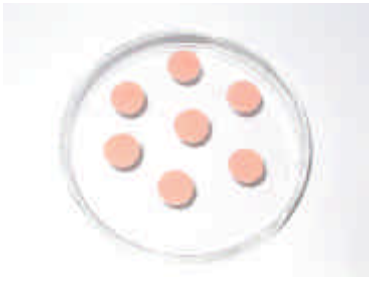
the flask, the Molloplast® B was pressed into the empty wax disk mold and the two flask compartments were closed again and maintained under continuous bench press pressure as recommended by manufacture. Curing was done by boiling the flask at 100 °C for 2 h. The following is the description of the preparation method:

1) Dental wax

2) Circular cutted wax

3) Dental flask

4) Molloplast® B-discs before curing



5) Molloplast® B-discs after curing

6) Molloplast® B-discs coated with CTS-films

Figure 14: Description of Molloplast® B-discs preparation method

2.3.4. Coating of Molloplast â B with chitosan films

1 g of chitosan samples of different average molecular weight (MW) and degree of deacetylation (DD) were dissolved in acetic acid solution (mass concentration of 1 %). Molloplast® B discs were dipped into chitosan solutions three times, left to air and dried for about 5 min. The biopolymer molecules formed a film on the surface of the Molloplast® B discs were finally fixed to the support by heating at 110 °C for 3-4 h. By this thermal fixation procedure bonds between chitosan molecules and the support were developed as discussed elsewhere [160-162]. The integrity and durability of each film obtained was tested by immersing it in artificial saliva for 24 h.

2.3.5. Microbial activity evaluation of chitosan films

Bacterial activity of each chitosan film formed on Molloplast® B discs was evaluated by measuring the dehydrogenase activity (TTC test-method) [144]. Molloplast® B discs coated with chitosan film and pure Molloplast® B discs (as control) were sterilized at 120 °C (autoclave). All discs were stacked into 40 ml SI-medium containing 2.5 x 10^4 Cfu/ml *E. coli.* All flasks were incubated by shaking at 37 °C / 200 rpm for several days (1-8 days). After each day, test and control discs were taken off each flask and immersed in sterilized test tubes containing 1 ml saline and 100 µl TTC (0.5 % w/v). All tubes were incubated at 37 °C for 20 min and finally centrifuged at 4000 rpm for 3 min. Then the red formazan was extracted with ethanol. After another centrifugation the red formazan was quantified by photometry at 480 nm.

2.4. Cylodextrin in drug delivery system

In this section ß-cyclodextrin (ß-CD) will be used as a drug delivery agent in the pharmaceutical and medical textile applications. Different antimycotic agents will be complexed with ß-CD. The solubility, hence, the antimycotic influence of the prepared complexes will be measured in-vitro using laser nephelometry in 96-well microtiter plate method.

2.4.1. Chemicals and materials

Sterile 96-well microtiter plates were kindly supplied by Greiner Bio-one GmbH, Germany. Breathe-Easy (Gas Permeable Sealing Membrane for Microtiter Plates 6x3.25 square inches were obtained from Carl Roth GmbH, Germany. CASY®cups, CASY® ton (isotonic dilution liquid for cell cultures), and CASY®clean were supplied by Schärfe System GmbH, Germany. McFarland standard Kit-0.5,1,2,3,4,5 and Sabouraud-Glucose-Agar with gentamycin-chloramphenicol (SGA) were from bioMerieux, Germany. ß-CD was purchased from Wacker-Chemie GmbH, Germany. Sabouraud-Glucose-Bouillon (SGB) was from Oxoid LTD., England. Phosphate buffered saline tablets (PBS), Econazole nitrate, Ciclopirox-olamine were from Sigma-Aldrich Chemical Company, Germany. Fluorescent fungal surface labelling reagent FUN® -1 cell strain (F-7030) (300 µl of 10 mM solution in anhydrous dimethylsulfoxide) and GH solution (Sterile 2 % D-(+) glucose containing 10 mM Na-HEPES, pH 7.2) were from MoBiTec, Germany. All other chemicals and solvents are of analytical grade.

2.4.2. Cell cultures

Two types of fungi were used in this study: *Candida albicans* DSM 11225 and *Candida krusei* ATCC 6258.

2.4.3. Preparation of cell cultures

Both of *C.albicans* and *C.krusei* were grown on SGA at 30 °C for 24-48 h. Three to five well-isolated colonies of the same morphological type were selected from an overnight culture using a sterile wire loop and inoculated in 20 ml SGB. The suspensions were incubated with shaking at 250 rpm/30 °C for 24 h. Then the overnight

cell cultures were counted using CASY® 1 and adjusted to a final working concentration of $6x10^5$ cells/ml in SGB.

2.4.4. Preparation of antifungal agents

Both Econazole nitrate (EC) and Ciclopirox olamine (CI) were independently dissolved in a mixture of chloroform/methanol 1:1 to achieve a final stock solution containing 20 mg/ml of antifungal agent. The stock solution of EC was diluted with SGB-medium and adjusted to be 1.25-100 µg/ml while as for CI it was in the range of 1.25-10 µg/ml. All solutions were stored at – 80 °C until used.

2.4.5. Preparation of inclusion and antifungal complexes

A solution of EC was prepared by dissolving it in a chloroform/methanol mixture 1:1. ß-Cyclodextrin (ß-CD) was dissolved in hot water at 85-90 °C. Equimolar amounts (1:1 molar ratio) of EC and ß-CD solutions were mixed together with stirring for 30 min at 85-90 °C [163]. By cooling, crystallization of the complex was obtained. The complex was filtered using G3 filter and kept in a desiccator overnight. On the other hand, the second complex between CI and ß-CD was also prepared according to the previously mentioned method, in which methanol was used as a proper solvent for CI [163]. Moreover, a molar ratio of 1:2 of CI: ß-CD was also used. The antifungal complex of ß-cyclodextrin-econazole nitrate complex (CD-EC) was prepared in a concentration range of 12.5-100 µg/ml using DMSO as a solvent, while the other complex ß-cyclodextrin-ciclopirox olamine complex (CD-CI) was prepared in a concentration range of 150-400 µg/ml using distilled water.

2.4.6. Phase solubility studies

The solubility of both drugs and complexes were studied and evaluated using also the laser nephelometry method described as before in section 2.1.2.
In this experiment both drugs and complexes were diluted in DMSO. Then the drug and complex solutions were independently pipetted into PBS-buffer with a concentration of DMSO of 1-5 %. All samples were scanned nephelometrically at 30°C, with an integration time of 0.1 s, so that a plate (96 samples) could be scanned in ~68 s. A gain of 122 and a laser intensity of 1 % were set to allow direct comparison of all re-

sults. All raw data were processed using the BMG NEPHELOstar Galaxy® Evaluation software. The scattered light will remain at a constant intensity until precipitation occurs. At that point it will increase sharply.

2.4.7. Preparation of microtiter plates

Microtiter plates were prepared with SGB as medium and $3x10^5$ cells/ml as inoculum. Using a multichannel pipette, 100 µl of 2 x antifungal concentrations was dispensed into columns 5-12 of sterile disposable 96-well microtiter plates. Column 5 and 9 contained the highest concentration and column 8 and 12 the lowest concentration of drug. Each concentration was repeated 8 times. Columns 3 and 4 (controls) received 100 µl of diluents SGB, columns 9-12 (blank; drug+medium) received also 100 µl of diluents. Then, 100 µl of working cells suspension prepared above were dispensed into each well of columns 3-8. The plates were covered by a gas permeable sealing membrane and scanned nephelometrically. The plates were shaken and incubated at 30 °C during measurement. The cell growth curve of each organism was obtained automatically every hour until 24 h in term of units. Then, by using the standard curve of *C.albicans* and *C.krusei* determined by McFarland standard kit, these units were calculated into cell counts/ml.

2.4.8. Staining with fluorescent dye (FUN-1)

To do this staining, overnight cell cultures of *C.albicans* DSM 11225 and *C. krusei* ATCC 6258 species were prepared. Cells of both cultures were counted and adjusted using CASY® 1 to a final working concentration of $3x10^5$ cells/ml in 20 ml SGB. This number of cells were inoculated with previously detected inhibitory and lethal concentrations of both complexes and without as positive control. These cultures were incubated at 30 °C for 24 h. Then 200 µl from each overnight cell culture was suspended in 1 ml GH solution. The suspension was centrifuged at 14000 g for 5 min. The pellets obtained were resuspended again in 50 µl GH solution. From the last suspension 10 µl was added to 10 µl of FUN-1 (10 µM). After incubation at 30 °C for 30 min 10 µl of cell suspension was trapped between microscopic slides in preparation for microscopy. All microscopy was carried out by means of the Olympus Fluorescence microscope. Epifluorescence illumination was provided by either a 50 or a

100W mercury arc lamp. An excitation filter of 480 nm and an emission filter = 530 nm were used. Photomicrographs were acquired with an Olympus Digital Camera. Photographic slides were digitalized electronically and composite figures were assembled from the resulting images with analysis software (Soft Imaging System®).

2.4.9. Instrumentation analysis

Cell counter

The cell count was determined using CASY® 1 Cell counter and Analyzer System Model TT , Schärfe System GmbH, Germany.

Fluorescence microscope

The FUN-1 stained fungi were observed using a Fluorescence microscope BX 40 in connection with a Digital Camera BX40+C5050 Zoom from Olympus Optical Co. GmbH, Germany, Analysis software: Soft Imaging System® GmbH, Münster, Germany.

GlpKa Instrument

Potentiometric titrations are performed using a GlpKa analyser (Sirius Analytical Instruments Ltd, East Sussex, UK). The protonation constants and the stability constants of each complex are calculated directly from the experimental data using the software package Refinement Pro (Version V1.114), Sirius Analytical Instruments, UK.

Differential Scanning Calorimetry (DSC)

Inclusion complexes, free drug, and ß-cyclodextrin were subjected to DSC studies using DSC (Model 2910, TA Instruments, Inc., GmbH, Germany). The temperature ranged between 20 °C and 400 °C, the scan rate was 20 °C/min. The sample weighed 3-4 mg.

Elemental analysis

Elemental analysis was carried out at the University of Duisburg-Essen using a Carlo Erba CH-1006 and an EA 3000 (Hekatech) elemental analyzer.

^{1}H-NMR-Spectroscopy

The ^{1}H-NMR-spectra were carried out at the University of Duisburg-Essen using a Bruker WM 300 (300 MHz) and Bruker WM Advance DRX 500 (500 MHz) (Fa. Bruker Analytik GmbH, Rheinstetten, Germany). The solvent used was d_6-DMSO with tetramethylsilane (TMS) as internal standard.

Chapter 3

Results and Discussion

3.1. Chitosan in textile applications

3.1.1. Introduction

An increasing demand develops for imparting active agents to textile materials (fabrics and non-wovens) by chemical ways in order to create additional properties (functional textiles). With synthetic fibres this may add a better hydrophilic behaviour (water retention, sweat transport etc.). On natural fibres this could mean the anchoring of bacteriostatic or odour binding agents and similar. An advantageous strategy is to rely on existing fibre types and to modify only the surface thus retaining the well known mechanical properties of the bulk fibre. In addition such a strategy imparts more flexibility to the textile finishing industry [164]. So an important task for research and development lies in the evaluation of methods how to anchor such biopolymers permanently onto fibre surfaces in a way that the biopolymers retain their bulk beneficial properties of action [165,166]. The physicochemical interactions used for permanent fibre surface modification are summarized in Table 8.

	Fibre type				
Modes of anchoring	**CO**	**WO**	**PA**	**PES**	**PAN**
Cross linker (resin finish)	Y	N	N	N	N
Ionic interaction	N	Y	Y	N	Y
covalent bonding	Y	Y	Y	N	N
Van der Waal Interaction	N	N	Y	Y	Y

Y: possible interaction N: no or weak interaction

Table 8: Interactions of biopolymers for permanent anchoring on fibres

One interesting scope for textile finishes lies in the hygienic area. The use of antimicrobial agents on textiles dates back to antiquity, when the ancient Egyptians used spices and herbs to preserve mummy warps. In 1935, Domagk, a German scientist, developed an important class of antimicrobial agents based on quaternary ammonium salts. In the last few decades, the prevention of microbial attack on textiles has become increasingly important to consumers and textile producers in order to create new fields of application. The major classes of antimicrobial agents for textiles include organo-metallics, phenols, quaternary ammonium salts, halogenated compounds and organosilicones. These finishes should be durable, have selective activity towards undesirable organisms, be compatible with other finishes and dyes, and be nontoxic to humans [167].

The antimicrobial activity of chitosan (CTS) against different groups of micro organisms, such as bacteria and fungi, has received considerable attention in recent years. Due to the variability of CTS preparations some of the antimicrobial tests performed up to now seem to be not conclusive so new testing methods have to be introduced. Some of them are described in this work too.

In this work, chemical bond formation via different anchor groups is compared regarding to add-on achieved and the antimicrobial activity of modified textiles exemplarily investigated. Given below are the results obtained along with appropriate discussion.

3.1.2. Effect of anchor groups on chitosan-add-on cotton

For permanent fixation of chitosan (CTS) on to cotton fibre, different anchor molecules were used for chemical bond formation. The anchoring amount varies since it is expected that only few anchoring points are tolerable in order to have high activity of the CTS polymer chain. But fixation points enough for giving permanent treatment have to be created. The reactive triazines were expected to give high fixation rates, the cyanuric chloride (CNC) possibly leading to more grafting yield than the methoxy-dichloro analogue (MCNC) as described in Figure 15 and Figure 16.

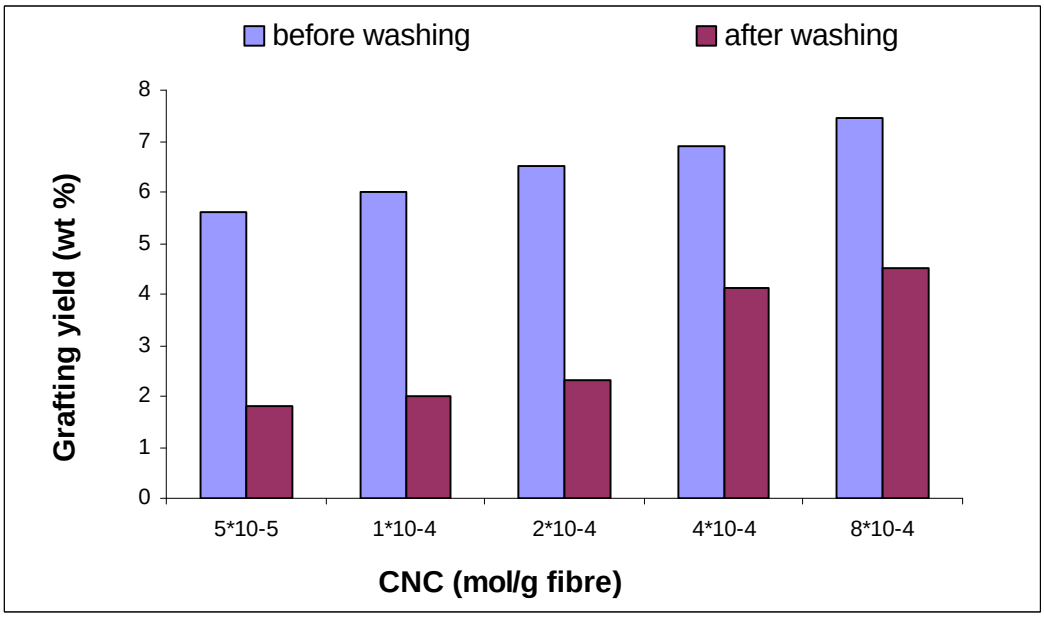


Figure 15: Effect of CNC on the grafting yield of chitosan-add-on cotton

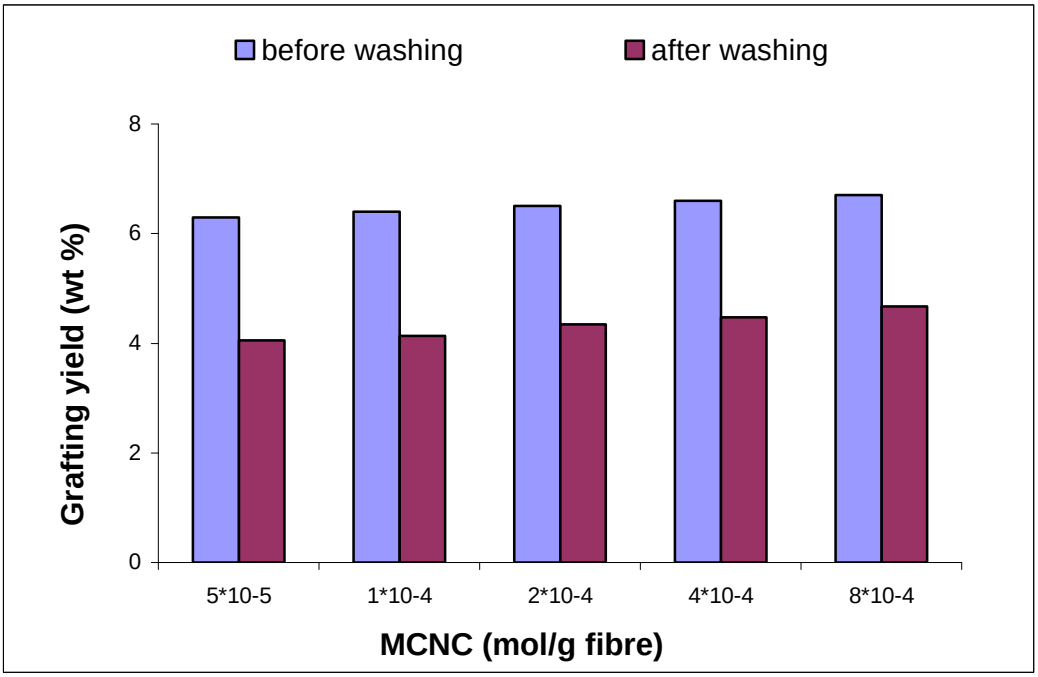


Figure 16: Effect of MCNC on the grafting yield of chitosan-add-on cotton

Propane tricarboxylic acid (PTCA) and butanetetracarboxylic acid (BTCA) were also used as anchor molecules because of their low toxicity. Both anchors bind via intermediate anhydride formation [168]. This intermediate would either react directly with cotton or form a cyclic anhydride of BTCA that would in turn react with cotton as described in Figure 17.

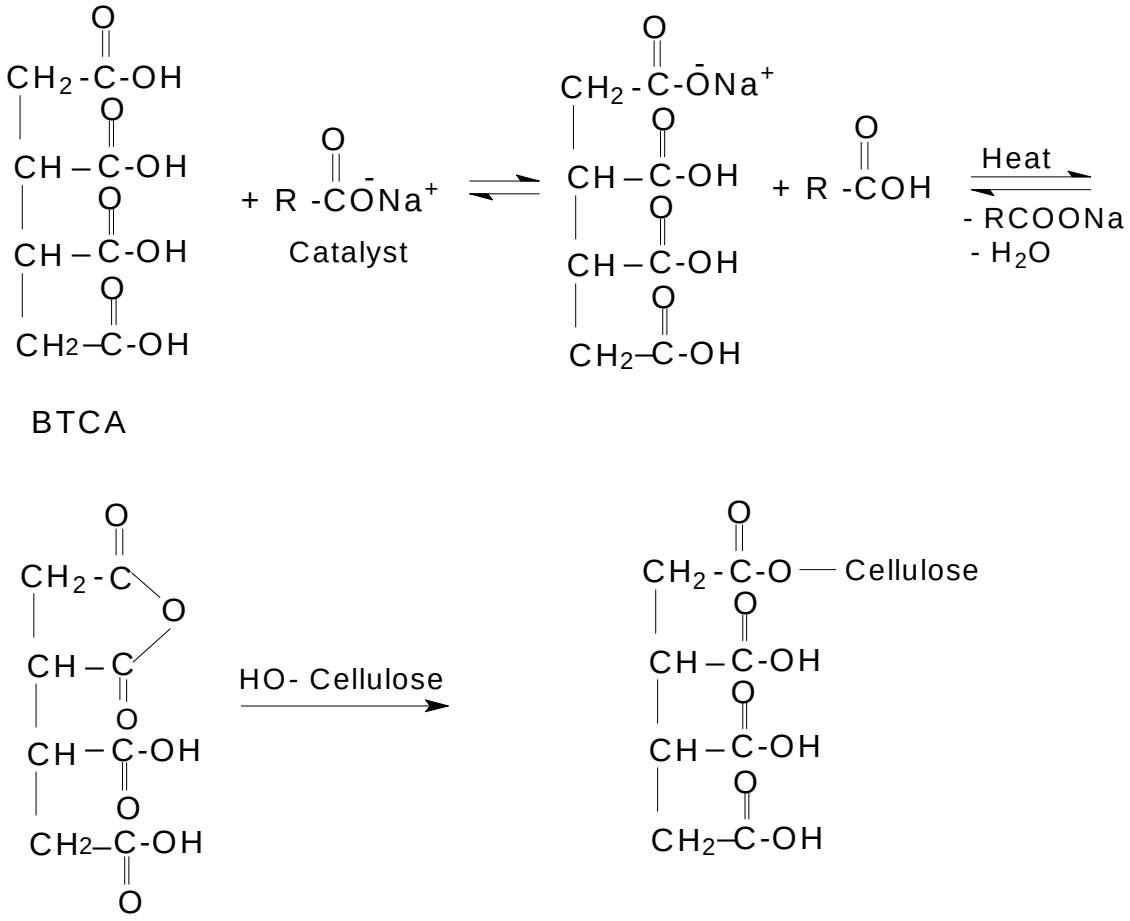


Figure 17: The mechanism of catalysis in the reaction of BTCA with cellulose [168]

It could be seen in Figure 18 and Figure 19 that a higher amount of anchoring chemical gives higher fixation values of CTS both directly after fixation and after washing as well. The relative loss on washing means removal of unbound CTS; is disappointingly high when using CNC under the fixation conditions described in Figure 15. In the other cases add-on after washing remains in practical ranges. It must be noted here that add-ons of > 5 % by weight of CTS with rather high MW as used means a very stiff product of low textile character.

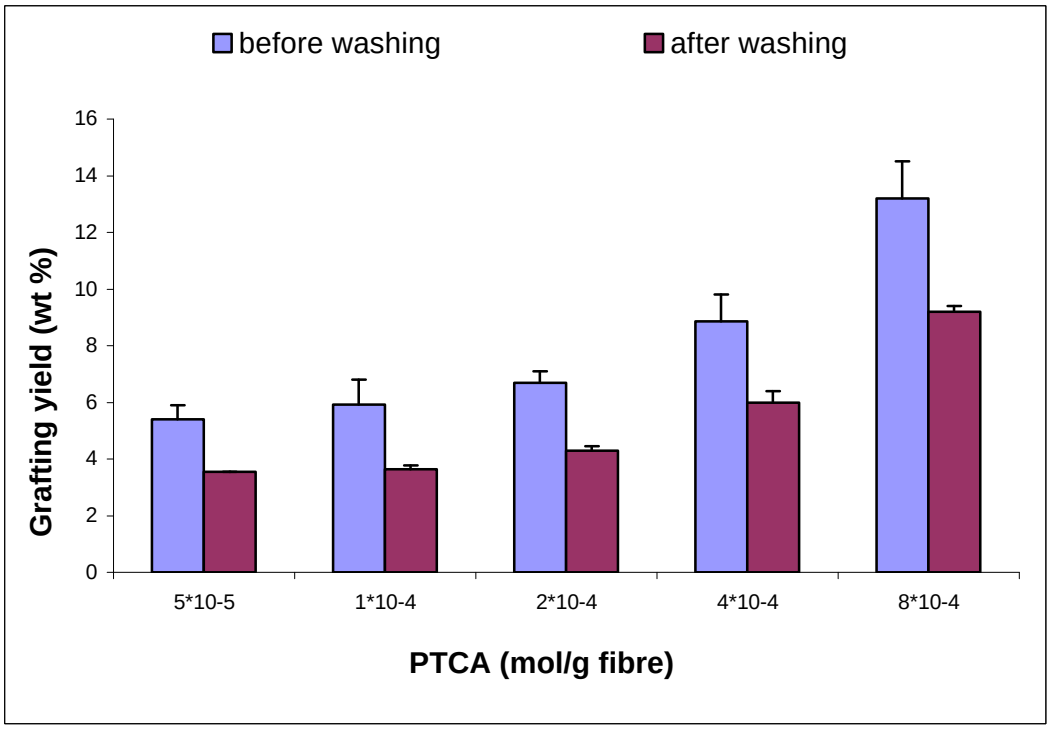


Figure 18: Effect of PTCA on the grafting yield of chitosan-add-on cotton

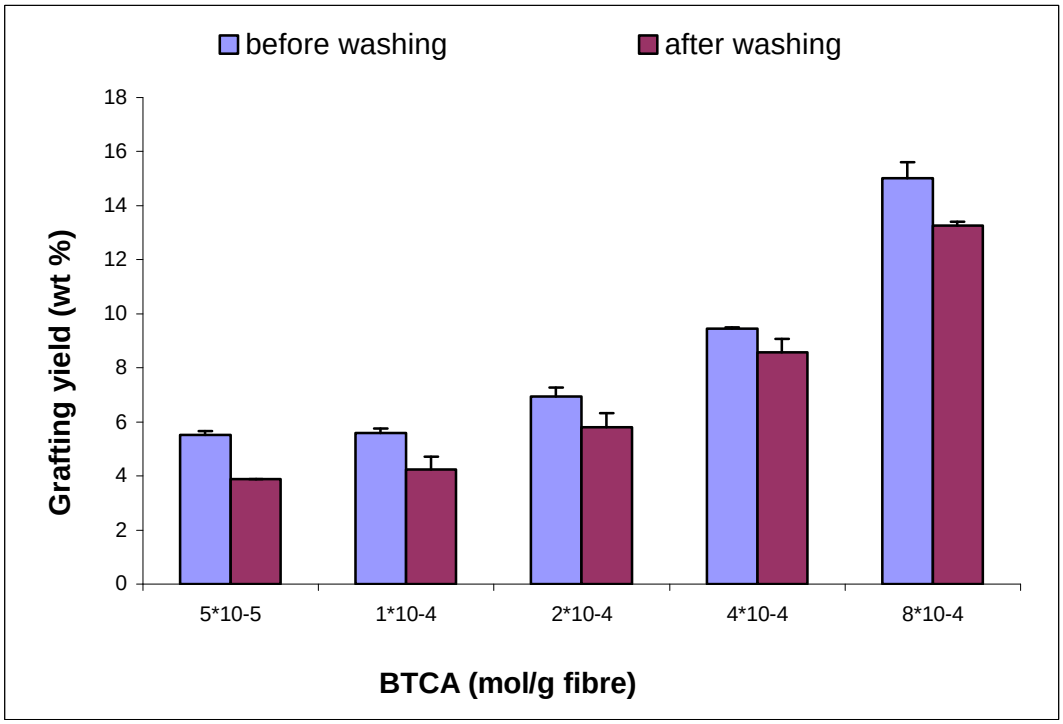


Figure 19: Effect of BTCA on the grafting yield of chitosan-add-on cotton

3.1.3. Polyelectrolyte titration of the treated cotton fabrics

Polyelectrolyte titration measurements on treated cotton fabrics may be used to assess the availabily of ionic charges on the cotton fabrics which give information on the (statistical) sites of crosslinker and on possible chain segment mobility of bio-polymer on-fibre. Chain mobility may determine any functionality envisaged [169]. The results represented in Table 9 and 10 show exemplarily that the use of the anchor group mainly cyanuric chloride, CNC or butanetetracarboxylic acid, BTCA almost only 1/3 of potential charge equivalents remain accessible after fixation onto textile. It can be shown that by varying the amount of anchor used, a higher charge accessibility can be reached with resulting in lower add-on. The charge accessibility related to the amount of permanent add-on can be increased strikingly using less anchor chemicals but with the risk of only low coverage and thus lower total binding capacity of the treated cotton fabric.

Grafting yield (in wt %)	1.8	2	2.3	4.5
Charges (Q) meq/g fabric	0.0076	0.004	0.0032	0.00140
Charges (Q) meq/g add-on	0.4222	0.202	0.1391	0.00032

Table 9: Polyelectrolyte titration results of cotton finished with chitosan using CNC anchor group

Grafting yield (in wt %)	3.4	5.3	8.7	13.5
Charges (Q) meq/g fabric	0.0020	0.0013	0.0003	0
Charges (Q) meq/g add-on	0.0858	0.0245	0.0038	0

Table 10: Polyelectrolyte titration results of cotton finished with chitosan using BTCA anchor group

3.1.4. Antimicrobial activity measurement of chitosan

Prior to anchoring experiments some antimicrobial investigations on CTS (He 85-60) dissolved in acetic acid at pH 6, 25 °C are evaluated using the so called quantitative suspension method [159] and turbidimetric method using laser nephelometry in microtiter plate [170]. It could be seen in Figure 20 and Figure 21 that CTS (He 85-60) has a pronounced influence against both bacteria and fungi at almost neutral condition.

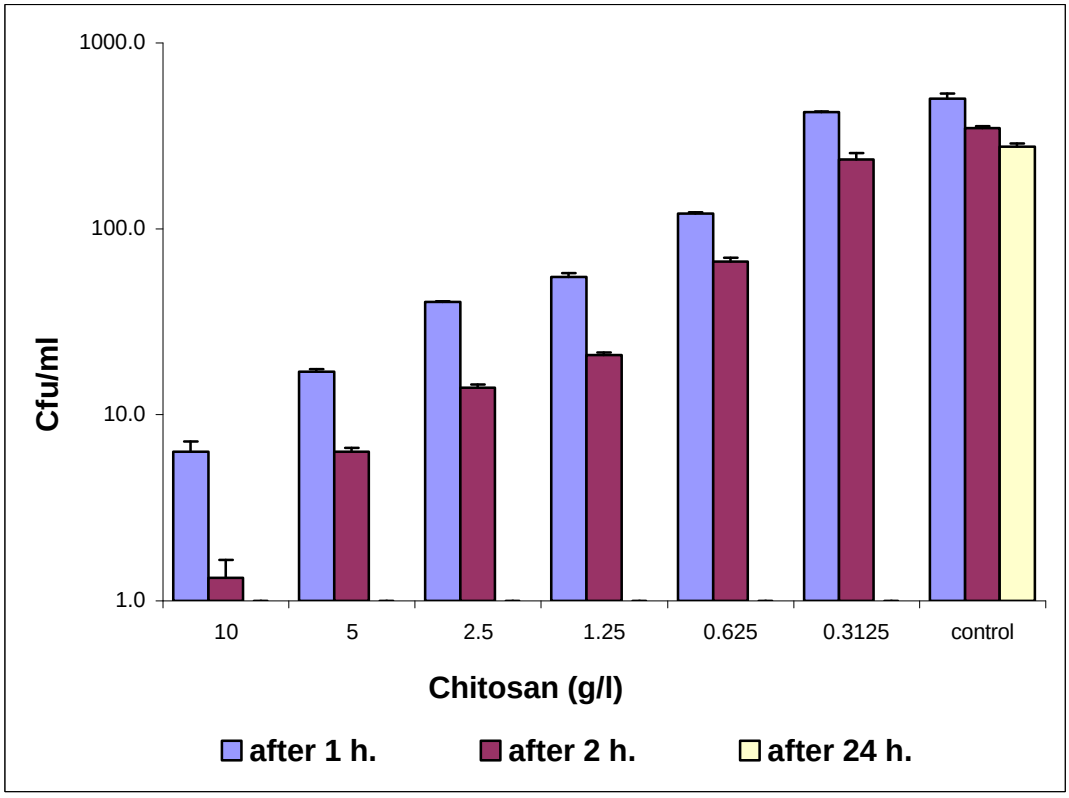


Figure 20: Effect of chitosan concentration on the reduction of *E.coli* at pH 6; 25°C

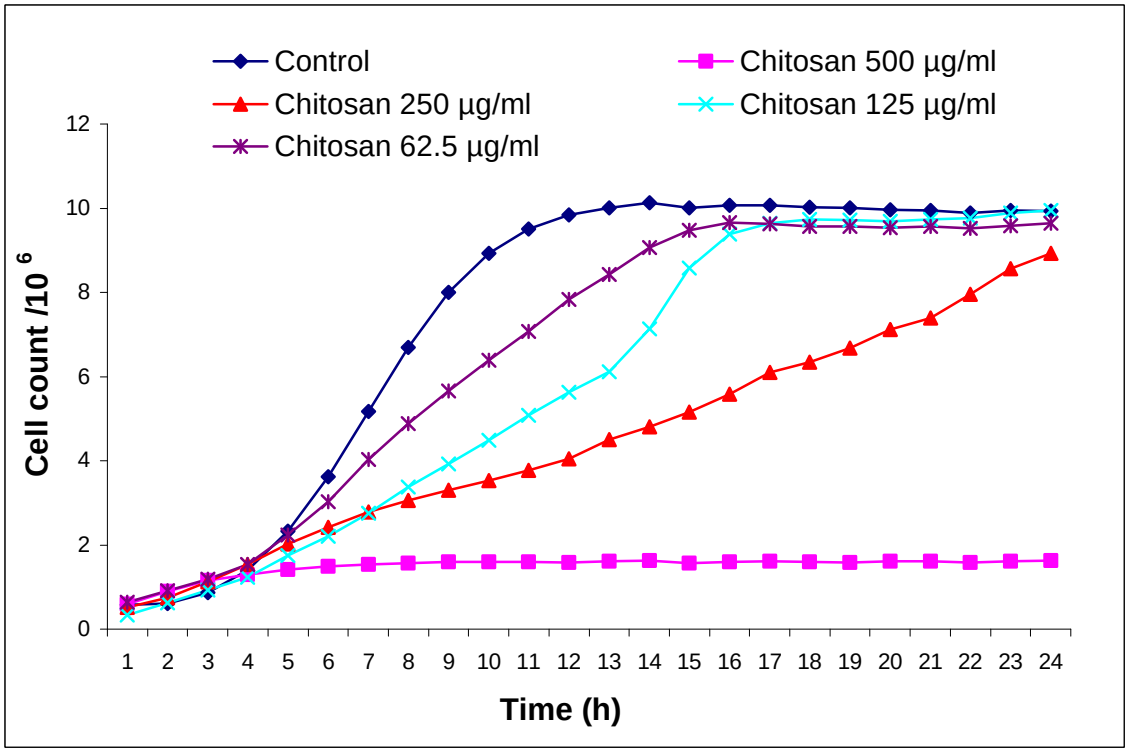


Figure 21: Effect of chitosan concentration on *Candida krusei*

3.1.5. Antibacterial activity measurement of cotton finished with chitosan

Cotton samples finished with chitosan (He 85-60) using different anchor groups were evaluated in their efficiency against bacteria (either gram-negative or gram-positive) by the so called TTC-test method [55]. This method serves as indicating system for the determination of the viability of micro organisms and can be used on surfaces. Figure 22 and Figure 23 give an example of antibacterial action of treated cotton fabrics after washing. It could be seen that by increasing the grafting yield of CTS the absorbance of formazan decreases, hence the antibacterial activity increases. Even if there is a distinct difference in grafting yield using the different anchors the antimicrobial suppression seems to be similar. As far as textile properties have to be retained add-ons of less than 5 % seem to be reasonable. It could thus be shown that chitosan imparts antimicrobial activity to cotton fabrics even when tested under nearly neutral pH-conditions. The finish will be durable because of chemical bonding. Future tasks will be aimed at testing against more microbe types and intro-

ducing chitosans of different molecular weight, the lower ones being preferred because of easy processing and also in the treatment of other polymer fibres.

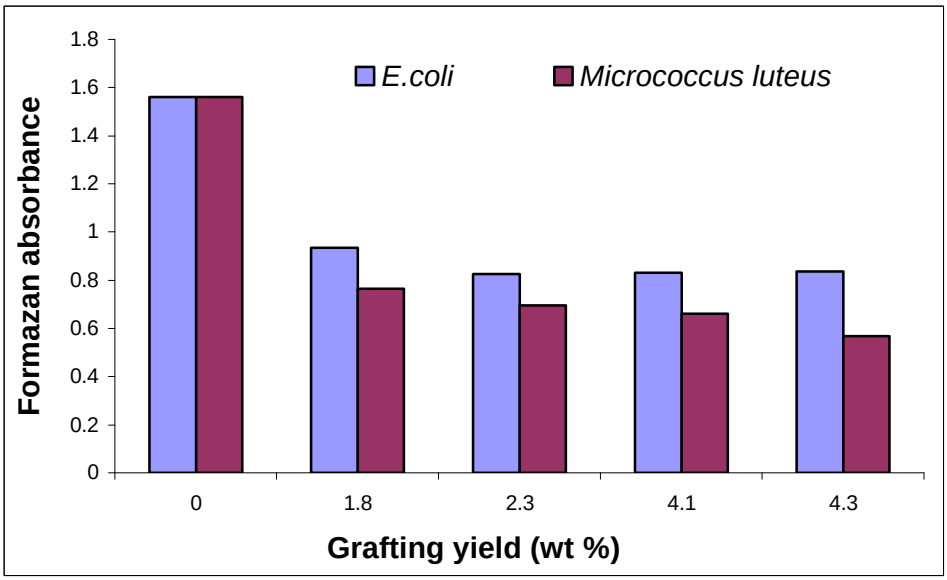


Figure 22: Effect of grafting of chitosan after washing on the reduction of bacteria; Anchor used: CNC

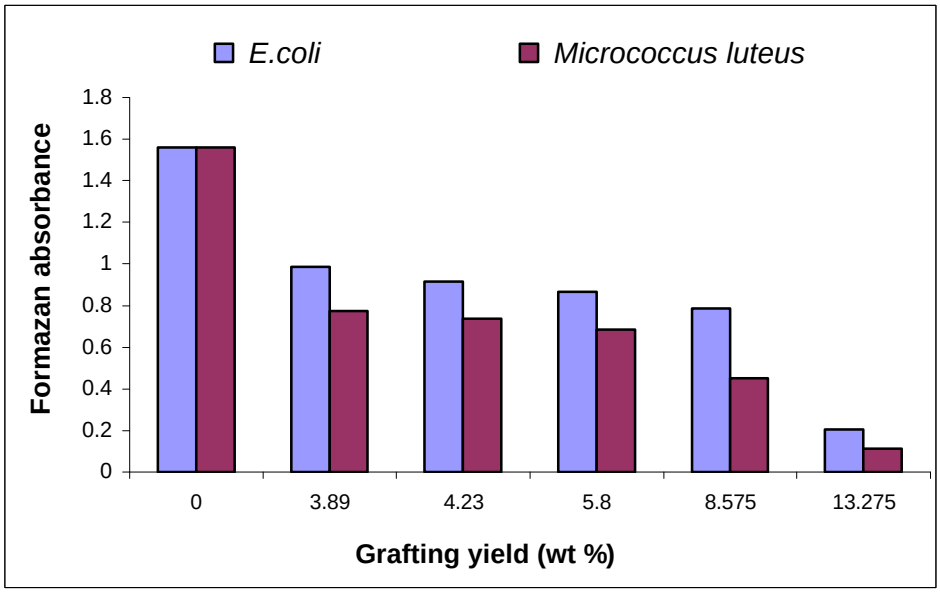


Figure 23: Effect of grafting of chitosan after washing on the reduction of bacteria; Anchor used: BTCA

3.1.6. Ionic crosslinking of cotton with CMCTS

3.1.6.1. Introduction

Carboxymethyl chitosan (CMCTS) was prepared by the chemical reaction of chitosan (CTS) with monochloro acetic acid under alkaline condition. In CMCTS both the 6-OH groups and/or the nitrogen atoms of the glucose amine units of chitosan are partially carboxymethylated. The *N*, *O*-carboxymethyl substituted derivatives are water soluble and contain an amino group either as primary ($-NH_2$) or secondary amine ($NH-CH_2COOH$) functions [171]. The degree of water solubility of this *N*- and *O*- substituted derivative appears to vary with the molecular weight of the material as well as the degree of substitution. The multifunctional chitosan derivative will be a favourable candidate to find new kind of polysaccharide with multifunction such as antibacterial character [172].

The textile industries continue to look for ecofriendly substances that can substitute toxic textile chemicals. In this point of view, CTS and CMCTS are selected to be used as excellent candidates for an ecofriendly textile chemical [173]. The major problem of CTS as antibacterial agent is its loss of cationic nature under alkaline condition as well as the antibacterial activity and due to this, carboxymethyl chitosan was used as a water soluble chitosan derivative used as antimicrobial finishing agent in textile application.

Our work based on the cationization of cellulose using the reagent Quab® 151 that imparts an ionic character to cellulose [174]. As a result, this cationic cellulose can absorb CMCTS as a polyelectrolyte of opposite charge to form ionic crosslinked cotton. It is assumed that such combination would enhance a wrinkle recovery angle of cotton, too. The cotton fabrics before and after finishing were monitored for wet and dry crease recovery angle. Tensile strength and elongation at break, K/S as enhancing dyeability [175] and antibacterial activity of the finished fabrics were evaluated.

The aforementioned cationized and cationized-CMCTS treated cotton were used for cotton finishing. The effects of application of such finishes on fabric performance as assessed by nitrogen percent, tensile strength, elongation at break, crease recovery, dyeing performance and antibacterial activity were studied. Given below are the results obtained along with appropriate discussion.

3.1.6.2. Characterization of carboxymethyl chitosan (CMCTS)

Carboxymethyl chitosan (CMCTS) was characterized by different spectroscopic methods as following:

FTIR-spectroscopic analysis

FTIR of carboxymethyl chitosan (CMCTS) (mixed with KBr as medium) shows broad absorption bands at 3463 cm^{-1} (NH_2,OH), 1735 cm^{-1} [(CO group), (carboxylic)] and at 1320 cm^{-1} (C-O stretching). Absorption at 1411 cm^{-1} was also observed.

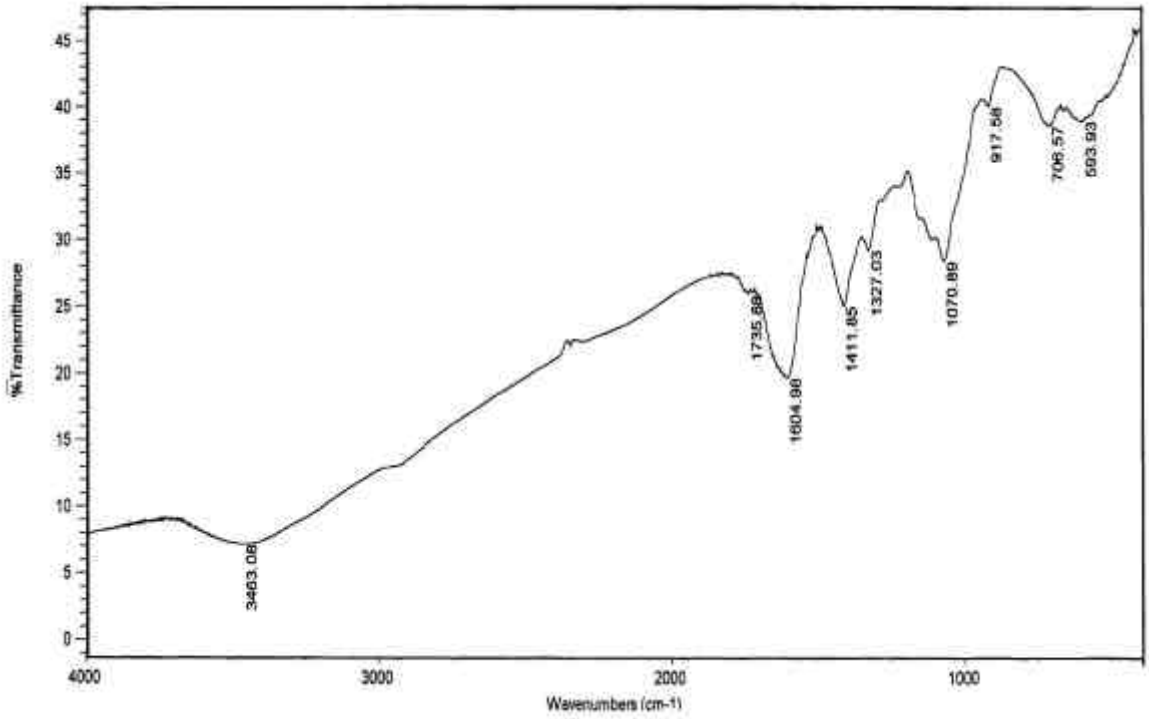


Figure 24: FTIR- Spectroscopy of carboxymethyl chitosan (CMCTS)

^{13}C-solid state- NMR spectroscopy

Carboxymethylation of chitosan (CTS) is achieved with monochloroacetic acid and sodium hydroxide. According to [176] this reaction takes place preferentially either at C-6 hydroxyl groups or at the NH_2-group resulting in *N*/*O*–carboxymethyl chitosan (CMCTS). The solid state ^{13}C-n.m.r. spectrum for a typical *N*-carboxymethyl chitosan shows signals attributed to the *N*-carboxymethyl substituent, at 47.7 and 168.7 ppm, for *N*-CH_2 and COOH, respectively [176].

But in case of our results, the solid state ^{13}C-n.m.r. described in Figure 25 shows signals at 73 and 175 ppm which attributed to –O-CH_2- and COOH respectively. This downfield shift of the carbon indicates the formation of *O*-carboxymethyl chitosan. The formation of this product agrees with the higher reactivity of hydroxyl group of C_6 in this heterogeneous reaction. The *N*-carboxymethyl substituent is not present because of the absence of peaks at 47 and 168 ppm for *N*-CH_2 and COOH respectively.

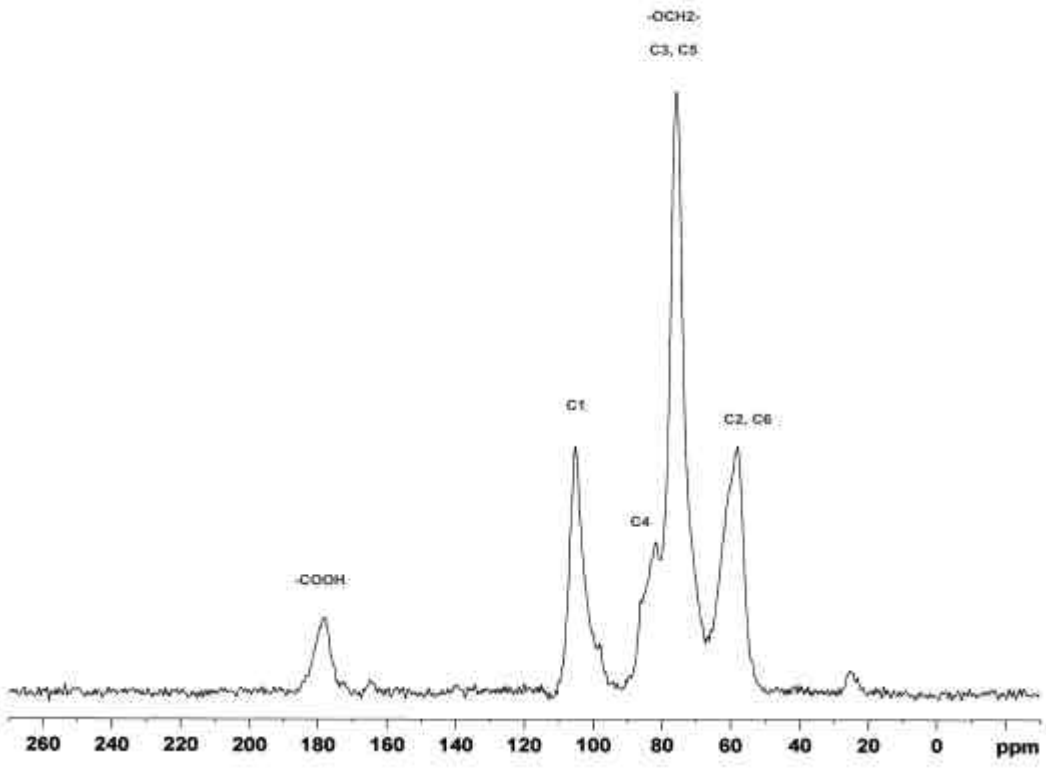


Figure 25: Solid state ^{13}C-n.m.r. spectrum typical for *O*-carboxymethyl chitosan

3.1.6.3. Effect of CMCTS (based on cationized cotton) on CRA

When a fibre is folded sharply the part of the fibre that is on the outside of the bend is stretched while the part on the inside of the bend is compressed. It would therefore appear that good recovery from creasing depends essentially on the ability to recover from large deformations. Thus it depends on the type of polymer and the manner in which the polymer chains are grouped together. Tendency to crease reten-

tion increases with increasing crystallinity: it increases in the series wool, silk, cellulose acetate fibre, viscose rayon and cotton. So fabrics made from cellulose fibres show poor crease recovery angle (CRA) and any improvement in this property would be most valuable. The increase in fabric CRA is strongly correlated with the increase in elastic recovery of the fibres and that fibres with higher modulus produce fabrics with greater resistance to creasing. Reaction of Quab® 151 with cotton fabrics produces cationized cotton as outlined in the following scheme reaction (Figure 26):

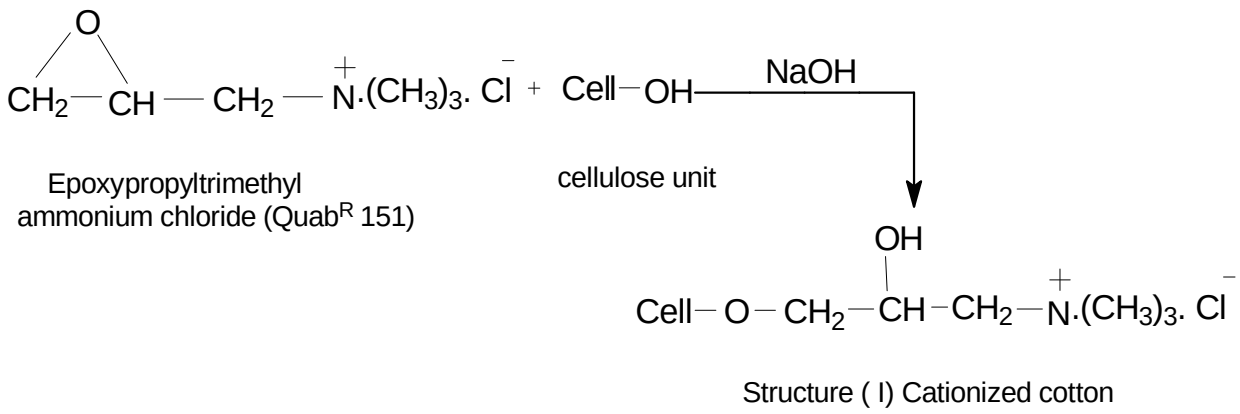
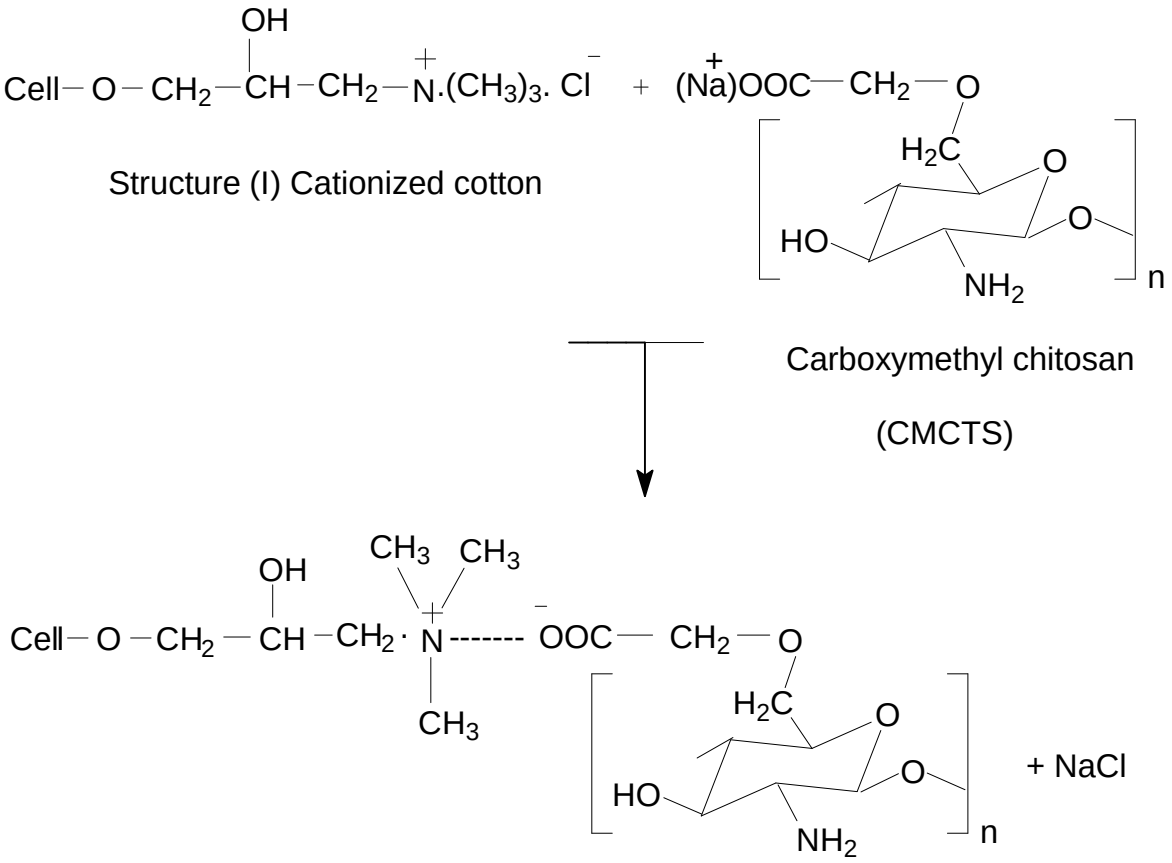


Figure 26: Reaction mechanism of Quab® 151 with cotton

Fabric samples treated with the aforementioned cationized finishes in absence and presence of carboxymethyl chitosan (CMCTS) were monitored for dry and wet CRA in the warp and weft directions. It is seen in Figure 29 that CMCTS finishes based on cationized cotton impart significant enhancement in wet and dry crease recovery of the cotton fabrics as compared with the cationized cotton described in Figure 28. Nevertheless, in presence of CMCTS, the enhancement in CRA is much greater than in its absence. This reaction was processed in water/acetone [153], because the solvent used has a pronounced effect on the cationized–CMCTS and the fixation percent. Cationized cotton bearing CMCTS may allow crosslinking if these groups are localized at two adjacent cellulose chains as suggested by structure II (Figure 27). The magnitude of crease resistance, whether wet or dry, would rely on the degree of crosslinks, the ratio of intra-inter-chain crosslinks and the state of the fabric during measurement of the crease recovery angle.



Structure (I) Cationized cotton

Structure (II) Ionic crosslinked cotton

Figure 27: Scheme of ionic crosslinking of cotton

The reaction of CMCTS with cationized cotton samples enhances both dry and wet CRA. This can be explained in the light of crosslinking due to ionic bonds between the single ended carboxyl groups and quaternary ammonium group found in cotton structure. It is obviously that at the same concentration of Quab® 151 (100 g/l) the dry and wet CRA are 198° and 230° but by the application of a CMCTS concentration of 40 g/l there is enhancement in dry and wet CRA to 210° and 265° and this is an improvement of the ionic crosslinking of cotton fabric.

Other conventional finishes of cotton are sometimes used in order to improve the CRA. These finishes involve the use of a thermosetting resin of the urea-formaldehyde type which is introduced into the fibre in the form of small molecules and condensed under strong acid conditions to form large molecules inside the fibre and lead to decrease of strength. In order to produce an improvement in CRA, it appears to be essential that the resin components used should be capable of forming a three-dimensional network within the fibre. The use of such resin in the finishing process

leads to the release of formaldehyde from the fibre surfaces which is environmentally toxic.

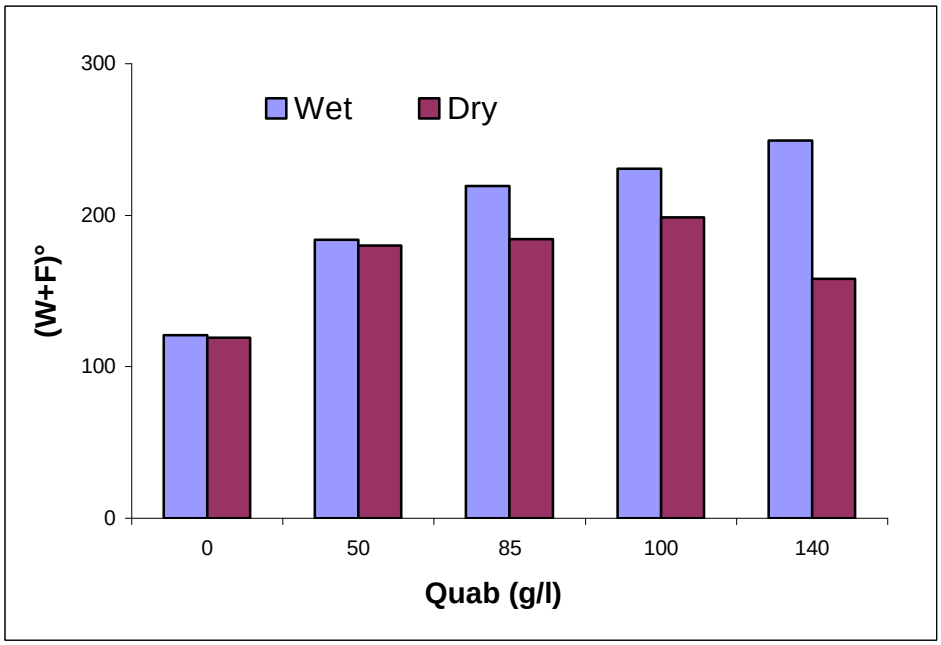


Figure 28: Effect of Quab® 151 on the CRA (W+F)

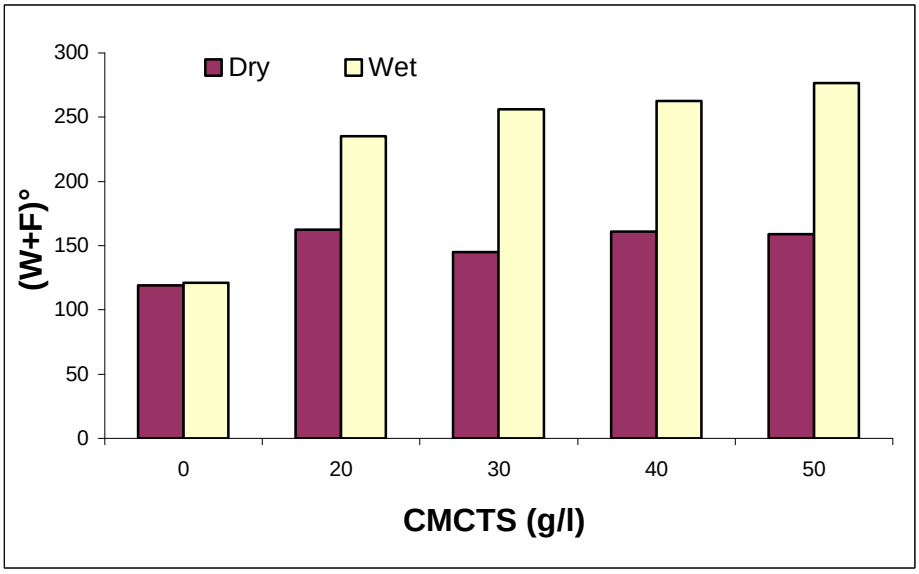


Figure 29: Effect of CMCTS (based on cationized cotton) on CRA

3.1.6.4. Effect of CMCTS (based on cationized cotton) on the strength properties

A certain minimum strength is necessary in any fibre to make a textile fabric. Because of this simple fact, a large amount of attention has been paid to measurement of the strength of fibres and to the effect of the method of manufacture, chemical treatment, etc. on this property. Much of the work done is empirical in nature because the strength of a material depends in a complicated way on the distribution of flaws or weak spots in the structure and not on the behaviour of the whole specimen. There are two main groups of factors that determine fibre strength, (I) constitution, fine structure and morphology and (II) test conditions such as relative humidity and temperature of the ambient air, rate or duration of loading, and test length.

Results of strength properties, including tensile strength (TS) and elongation at break (E%) of cotton fabrics finished are shown in Table 11. Obviously, carboxymethyl chitosan (CMCTS) (based on cationized cotton) improves the tensile strength of the cotton fabric most probably due to the extra strength of CMCTS film. It is clear that pre-cationization of the ether crosslinked cotton samples leads to slight improvement in the tensile strength (TS) as well as elongation at break (E%). Such improvement may reflect the cationization effect on the post-etherifies cationized cotton according to ref. [177] and/or the ionic crosslinking nature as it may bring the cellulose chain closer to each other, hence, an increasing in the TS is observed. For example the tensile strength increases from 24.5 cN for untreated fabric to 32.4 cN for fabrics treated with cationized CMCTS. Such differences in the values of tensile strengths could be associated with differences in the strength of the films of the chitosan finishes which, in turn, is governed by molecular weight of the chitosan finish, nature and distribution of its substituents and mode of association of the finish with the cotton fabrics. Table 12 shows also that the elongation at break is not significantly affected when the fabric was treated with the cationized-CMCTS based finishes. The elongation at break displays a value ranging between 12 and 18 % when the finishing was carried out using the cationized based finishes in presence of CMCTS.

Quab® (g/l)	TS (cN)	E %
0	24.5	12.0
50	24.5	13.3
85	24.0	13.1
100	23.5	12.3
140	23.5	12.4

Table 11: Effect of Quab® 151 on the strength properties of finished cotton

CMCTS (g/l)	TS (cN)	E %
0	24.5	13.3
20	24.7	14.9
30	25.1	15.2
40	28.0	18.7
50	32.4	18.4

Table 12: Effect of CMCTS (based on cationized cotton) on the strength properties

3.1.6.5. Effect of ionically crosslinked cotton on the dyeability

The technique of dyeing textiles can be traced back to prehistoric times. When a fibre is immersed in dye bath, dyeing principle takes place in three stages, namely: (a) transportation of the dye from the solution to surface of the fibre, (b) adsorption of the dye at the fibre surface and (c) diffusion of the dye from the surface to the interior of the fibre. The stage (a) is governed by the movement of dye liquor relative to individual fibres. The remaining two stages depend on the nature of the fibre and the dye molecule. Some or all of the following forces will contribute to the total adsorption: (1) electrostatic attraction between charged sites in the substrate and ionic

substances; (2) attraction by induction between ionic substances and a neutral substrate; (3) hydrogen bonds; (4) non-polar Van der Waall's forces and, (5) chemical bond formation. In textile finishing, dyeing of finished fabrics is important to evaluate and explain the effect of these finishing chemicals on the dyeing performance. Colour measurement is mainly used as an aid in the development of formulations and for the measurement of undesired colour variations of the textile materials.

Figure 30 and Figure 31 show K/S value (measure of colour strength) of cationized cotton fabric before and after crosslinking with CMCTS. It is seen that increasing of Quab® 151 concentration from (50-140 g/l) enhances K/S values of dyed samples and this finding is considered as an evidence for the cationization of cotton fabrics. It is also seen that for a given Quab® 151 concentration and with increasing CMCTS concentration there is decrease K/S values of the dyed samples whether with reactive or acid dye; both have the same trend. From this it is obviously that the K/S values depend largely on the magnitude of both cationization and concentration of CMCTS. Also, CMCTS may blocks the site of dye-uptake.

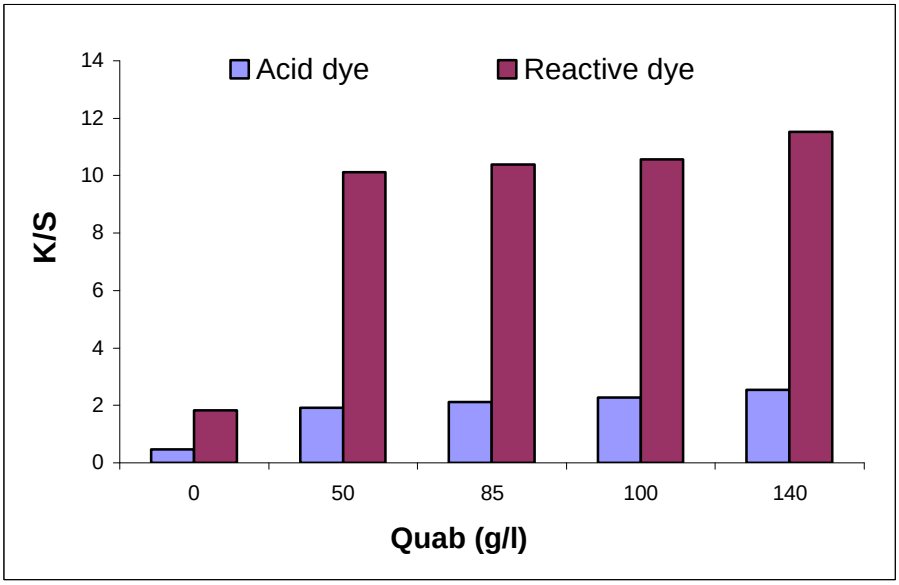


Figure 30: Effect of Quab® 151 on the dyeability of cotton fabrics

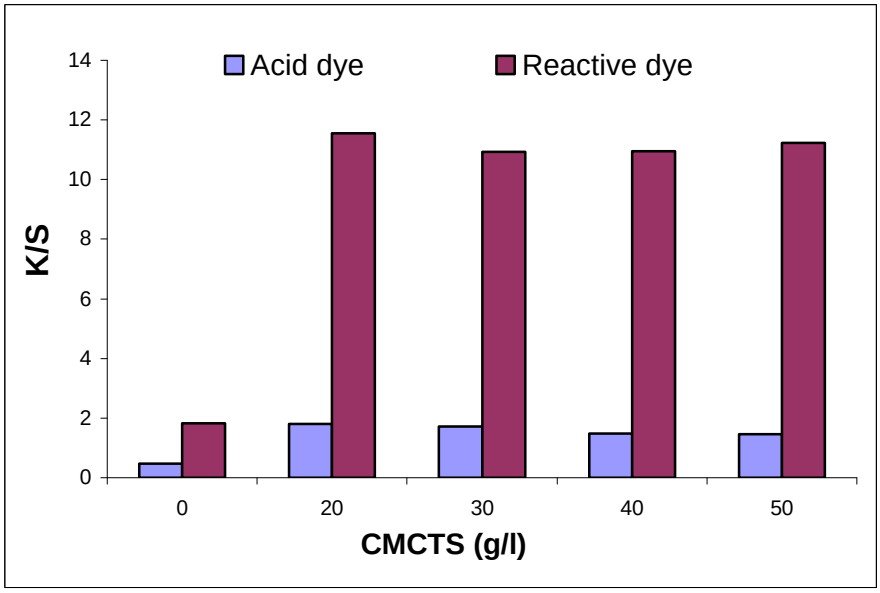


Figure 31: Effect of CMCTS (based on cationized cotton) on the dyeability of cotton fabrics

3.1.6.6. Nitrogen content (%N)

The reaction of modified CTS with cotton cellulose can be traced through monitoring the nitrogen percent of the finished fabrics. This indicates that the carboxymethylation of CTS exert no significant effect on its interaction with cotton cellulose. The results found that there was no significant difference between N % for the cationized cotton fabrics or for cationized CMCTS based cotton fabrics.

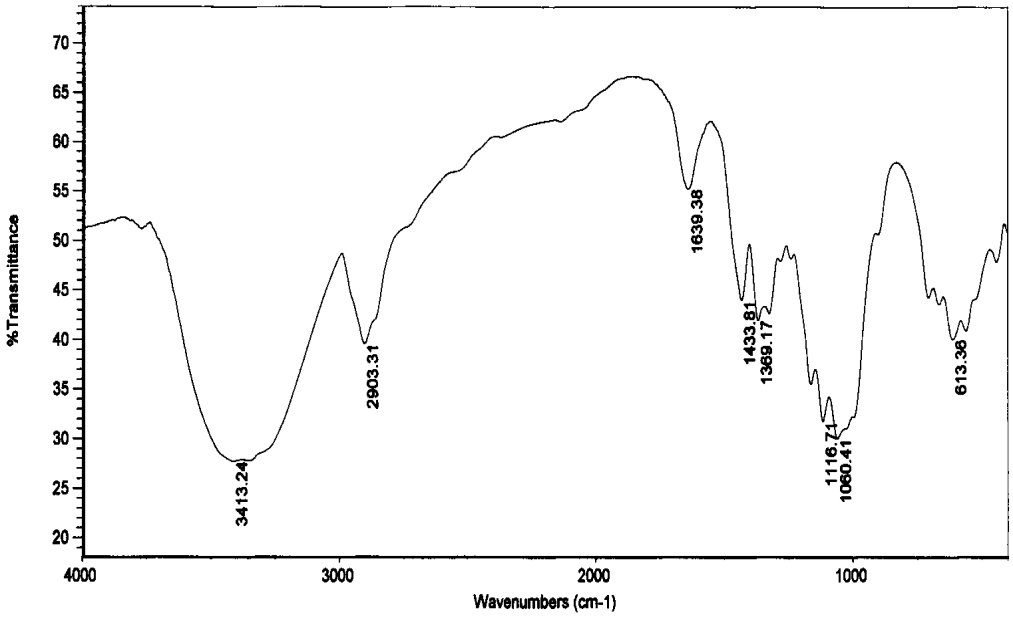
3.1.6.7. FTIR-analysis of treated cotton fabrics

Figure 33 (a,b,c) shows the FTIR spectroscopy of bleached cotton (a), cationized cotton (b) and cationized cotton after treated with CMCTS (c). The three spectra of these substrates depict common bands which can be summarized as follows:

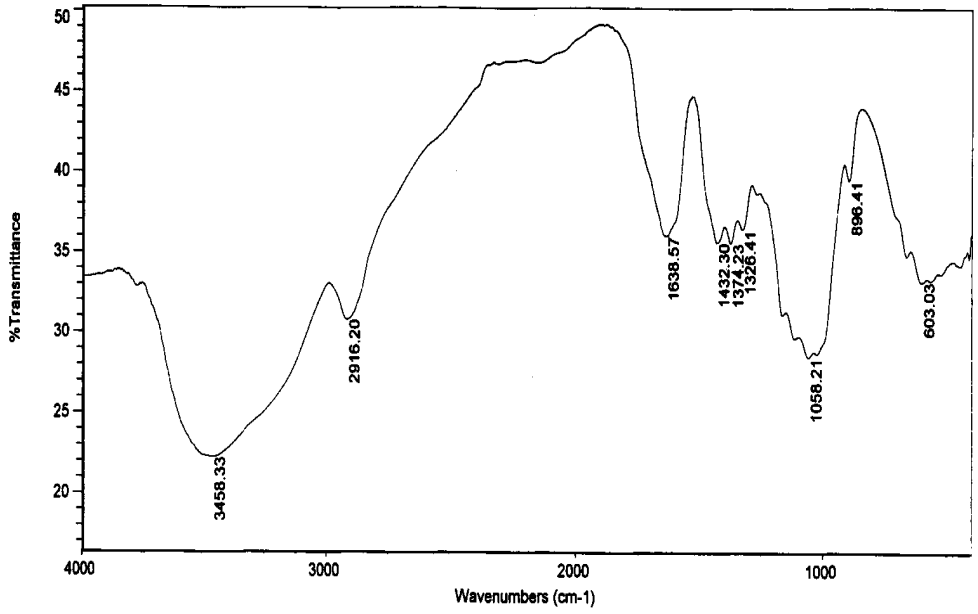
(a) A broad strong peak at 3400 cm^{-1} and medium beak at 612 cm^{-1} are attributed to stretching and out of plane bending of alcoholic –OH.

(b) Medium peak at 2900 cm^{-1} and 1430 cm^{-1} are attributed to asymmetric stretching and scissoring, respectively, of -CH_2 groups. A weak peak observed around 896 cm^{-1} is attributed to stretching of C-$N^{(+)}$ bond formed in cationized cotton (b). This peak is neither observed in the spectra of bleached cotton or CMCTS.

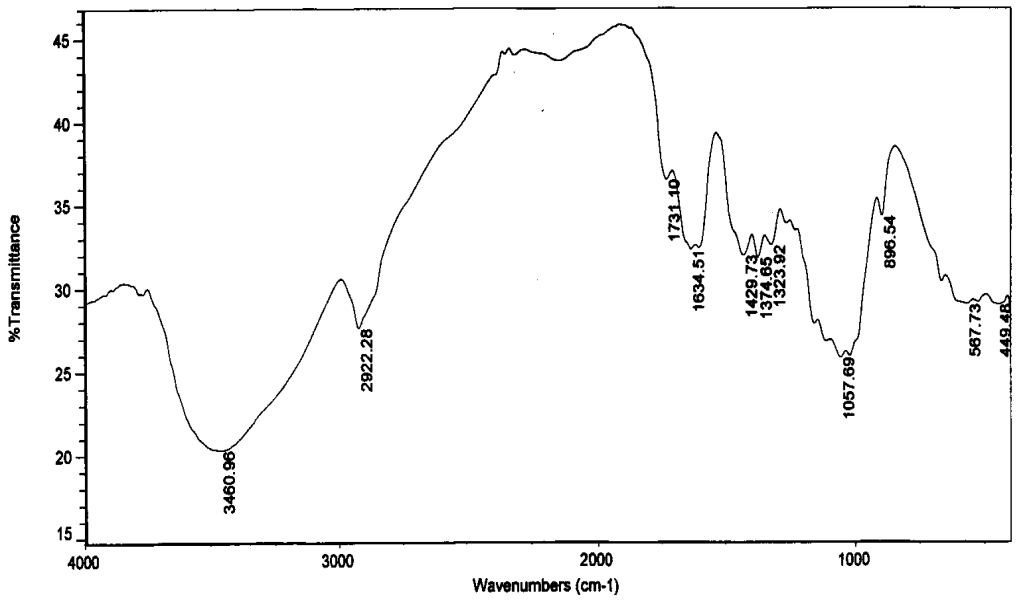
(c) Weak peak at 1730 cm^{-1} is attributed to stretching of carboxylic carbonyl groups. Also the weak peak at 896 cm^{-1} represents the $C\text{-}N^{(+)}$ of cationized cotton.



(a) FTIR of bleached cotton fabric



(b) FTIR of cationized cotton fabric



(c) FTIR of CMCTS based on cationized cotton

Figure 32: FTIR- Spectroscopy of : (a) bleached cotton; (b) cationized cotton; (c) CMCTS based on cationized cotton (all milled with KBr)

3.1.6.8. Effect of CMCTS (based on cationized cotton) on wettability

Wettability measurement for different types of textile materials is important in order to evaluate the hydrophilicity of textile materials. This property affects the quality and hence the marketing of such textile materials. Wettability plays an important role in dry and wet textile impregnation processes. Different methods of finishing techniques are taken into consideration in order to improve the wettability of cotton fabrics, consequently the hydrophilic properties.

In this study, cotton fabrics treated with the aforementioned cationized finishes in absence and presence of CMCTS were monitored also for wettability measurements. The results obtained are shown in Figure 33 and Figure 34. It is seen that CMCTS finishes based on cationized cotton impart significant enhancement in wettability of the cotton fabrics as compared with the cationized cotton. Nevertheless, in presence of CMCTS there is a synergistic enhancement in wettability than in its absence (Figure 34).

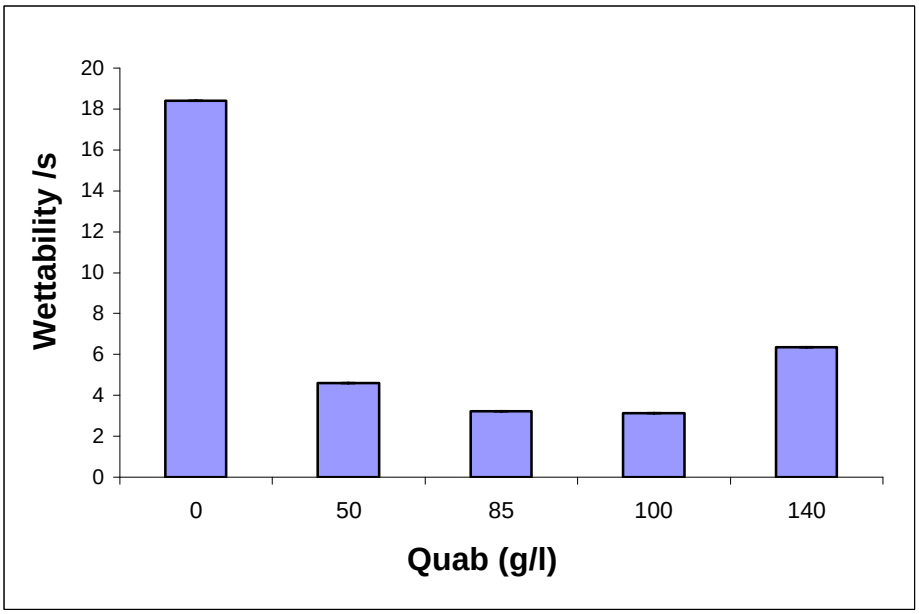


Figure 33: Effect of Quab®-treatment of cotton on wettability

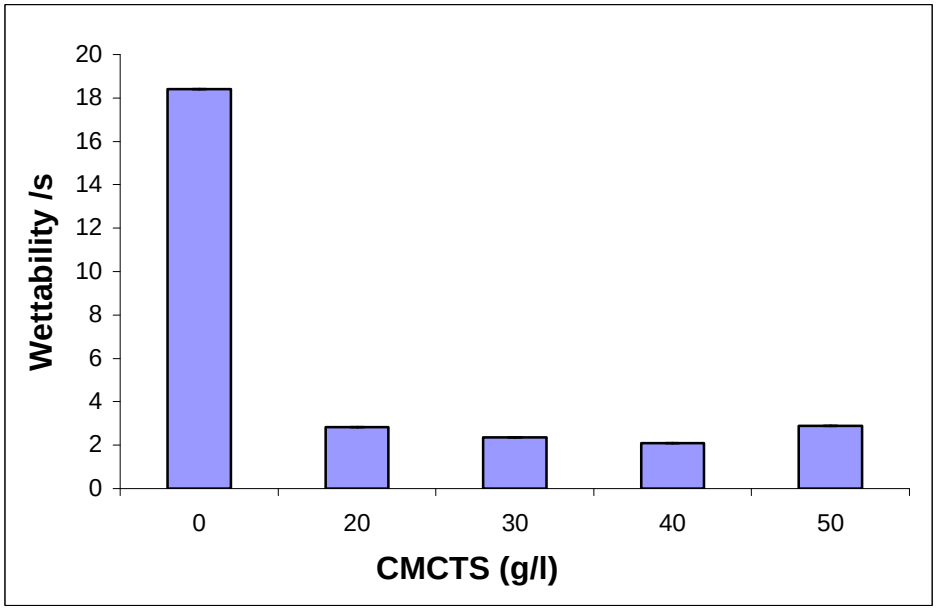


Figure 34: Effect of CMCTS (based on cationized cotton) on wettability

3.1.6.9. Effect of CMCTS (based on cationized cotton) on the antibacterial activity

Cotton fabric with good antibacterial activity is obtained by cationization of cotton using Quab® 151 in the presence of alkali. Different concentrations of Quab® 151 were applied to the cotton fabrics using the pad-dry-cure method. The antibacterial activity of the treated cotton fabrics were evaluated against *E.coli* and *Micrococcus luteus* according to the TTC–test method [55]. In Figure 35 the absorbance of formazan is directly proportional to the number of active cells. Figure 35 shows that the activity of the cells of both micro organisms decreased by increasing the concentration of Quab® 151 in comparison with the control, hence, the antibacterial activity of the treated cotton fabrics increased. The antibacterial activity of CMCTS (based on cationized cotton fabrics) were evaluated and the results below in Figure 36 shows that the activity of the cells decreased by increasing the concentration of CMCTS based on cationized cotton. This finding results confirm structure (II) found in Figure 27.

There are two proposed mechanisms of antibacterial activity by chitosan (CTS). In one mechanism, the polycationic nature of CMCTS based on the amino groups present in CTS molecules in addition to the permanent positive charge arised from the cationized cotton, both interact with the negative charged residues present at the cell wall of bacteria leading to alteration of the cell wall permeability, consequently, interfere with the bacterial metabolism and result in the death of cells [178]. Another mechanism assumed that the antibacterial activities of chitosan derivative can also be closely correlated to the formation of hydrophobic microareas. At pH 7 the degree of protonation of NH_2 is very low and thus the repulsion of NH_3^+ is weak, so the strong intermolecular and intramolecular hydrogen bond results in the formation of hydrophobic microareas in the polymer chains [179]. At the same time, the carboxyl groups in the polymer chains are strongly hydrophilic. Therefore, the polymer chains have hydrophobic and hydrophilic parts. This amphiphilic structure provides structure affinity between the cell walls of bacteria and the chitosan derivative. On the other hand, carboxylate groups having strong polarity can react with phospholipids in the cell membranes and the nonpolar parts of the derivatives can insert the hydrophobic areas. The cell membranes will be broken because of the strong electrostatic interaction and result in the death of cells. The results was compared with other results of cotton fabrics finished with quaternized chitosan. It is found that the antibacterial efficiency increased

in case of cotton finished with quaternized chitosan than with chitosan alone. This finding prove that the former mechanism should be considered to be closer to the real mechanism.

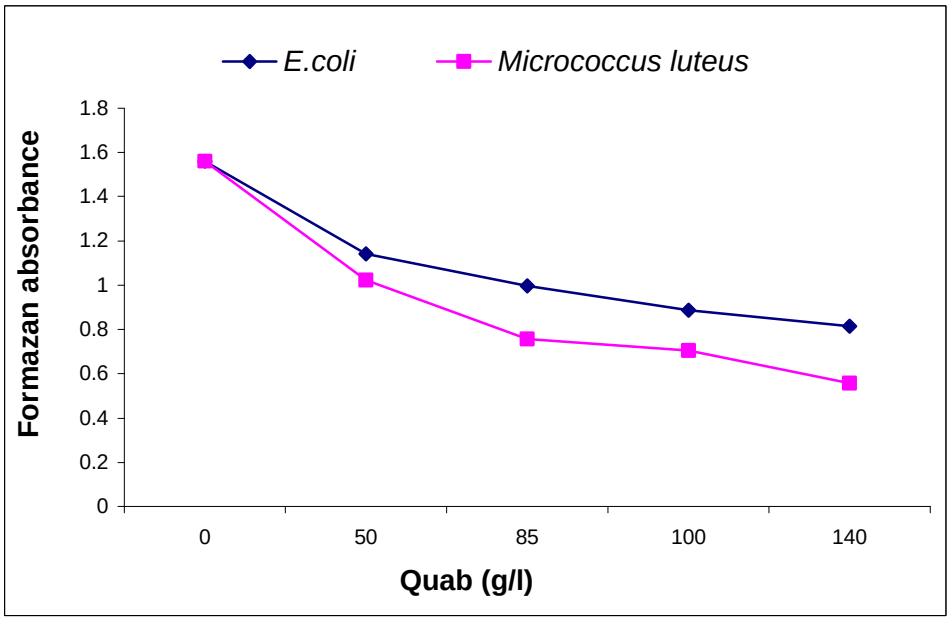


Figure 35: Bacterial activity on Quab®- treated cotton

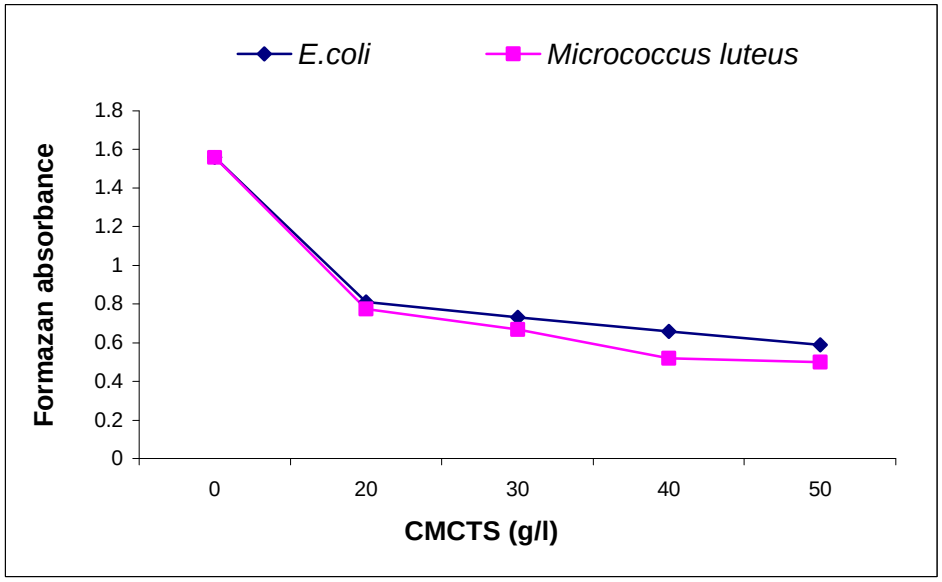


Figure 36: Bacterial activity on CMCTS- treated cotton

3.2. Chitosan in Dentistry

In this part chitosan will be used as antimicrobial coating agent on Molloplast® B-soft liner, denture based material in order to treat denture stomatitis.

3.2.1. Introduction

Denture soft liners are commonly used for relieving pain, tenderness and rendering dentures more comfortable particularly under oral conditions accompanied with thin atrophied oral mucosa which is commonly observed for elderly patients. These patients are suffering from inability to masticate foods, this makes them diet dependent patients, shifting them to high-calories soft foods like carbohydrates. These oral conditions favour bacteria growth and hence disturb the normal oral bacteria symbiosis, favouring flourishing of pathogenic micro-organisms, and result at the end in a disease termed as denture stomatitis.

Soft lining materials are viscoelastic materials used to form all or part of the fit surface of a denture. Soft lining materials are used on dental prostheses to aid in the distribution of functional loads to the denture bearing area, to avoid localized stress concentration and to improve retention by engaging undercuts [180]. Resilient soft liners have been a valuable assist for dentists because of their visco-elastic properties. They act as shock absorbers, reduce and distribute the stress on the denture bearing tissue avoiding local stress concentrations and providing comfort for patients during mastication [181-183]. The materials currently available are divided into operationally defined groups, tissue conditioners, functional impression materials and soft liners for short-term or long-term use. The materials are also classified according to polymer composition [184]. Some soft denture liners maintain their softness and performance for several weeks, others for up to 10 years [184].

According to Philips in 1991, the commercial soft liners are self-curing or heat-curing materials, either from plasticized acrylic resin, silicone rubber, silicones or vinyl resins [185]. The heat-cured material was described as being made from a poly (ethyl methacrylate) polymer and n-butyl ester monomer with up to 30 % phthalate plasticizer. In the self cured material poly (ethylmethacrylate), poly (methyl methacrylate) or an acrylate co-polymer is mixed with an aromatic ester-ethanol liquid. Recently, new materials such as fluoropolymer resin and polyphosphazine rubber have become available [186].

Beyond the fully synthetic polymer materials several naturally occurring biopolymers, e.g. chitosan (CTS) and its homologues, came into the focus of interest. At slightly acidic conditions chitosan is a polycationic polymer, possesses reactive amino groups with antimicrobial properties and can be used as bioadhesive polymer for oral mucosal delivery and surgery [187-189]. CTS in this study was evaluated as antimicrobial coating agent on Molloplast® B soft liner (heat-curing materials) in order to prevent denture stomatitis.

Here denture stomatitis is the most common form of oral candidosis [190]. One reason for that is; the fitting surface of the denture can act as a reservoir of *Candida albicans* [191,192]. The presence of *Candida albicans* on the upper fitting surface of the denture is a major causative factor in denture associated chronic atrophic candidosis. If the denture stomatitis will be reduced or even prevented by medical grade biopolymers, numerous prerequisites has to be fulfilled: The biopolymer should be easy to handle, effective in the removal of organic and inorganic deposits, bactericidal, fungicidal, non-toxic to patients and compatible with the complete denture materials [193].

In this study we focussed our interests on pathways to develop potentially biocompatible soft denture liners coated with chitosan films that need not to be removed and of course can deliver medication to treat denture stomatitis. Hence we started our experiments with the evaluation of the antimicrobial activity of several chitosans of different origin, MW and DD either dissolved in acetic acid (pH 6) or immoblized film on the surface of Molloplast® B soft liner.

3.2.2. Effect of chitosan on the reduction of bacteria

At first the effect of concentration of dissolved chitosan (CTS) on the number of bacteria at 25 and 37 °C by the so called quantitative suspension method was investigated. The antibacterial potential of dissolved chitosan (He 85-500) solution (1 %, pH = 6) at 25 °C is exemplified in Table 13. These conditions have been chosen because some work in the literature presumably has only measured the effect of standard acetic acid solvent on bacteria. Here it can be seen that at high dilution the inhibitory effect of the chitosan used on *E.coli* is valid under almost neutral conditions. It is clearly visible from Table 13 that the number of colonies was reduced by chitosan from 10^4 to = 10^2 Cfu/ml only after 24 h incubation time. If the temperature was raised

from 25 °C to 37 °C the antibacterial activity of CTS (He 85-500) is higher as described in Figure 37.

Chitosan (g/l)	**10**	**5**	**2.5**	**1.25**	**control**
Cfu/ml * 10^2 (1h)	192 ± 8.5	247 ± 2.1	266 ± 6.2	280 ± 3.3	360 ± 8.2
Cfu/ml * 10^2 (2h)	140 ± 4.1	145 ± 4.1	190 ± 8.2	210 ± 8.2	285 ± 4.1
Cfu/ml (24h)	= 100	= 100	= 100	= 100	260 ± 8.2

Table 13: Effect of chitosan concentration on the number of *E.coli* at 25 °C, pH 6

The results obtained can be interpreted in terms of an interaction between positive charged chitosan molecules and negative charged residues on the cell surface as discussed in details in the introduction chapter (1.3.9). Further evidence for the protonation mechanism is coming from experiments with solutions of modified CTS [194]. By introducing quaternary ammonium salts onto the backbone of CTS the antimicrobial activity as well as the water solubility of modified CTS increased [194]. This finding proves that the antimicrobial activity of CTS based on its cationic nature.

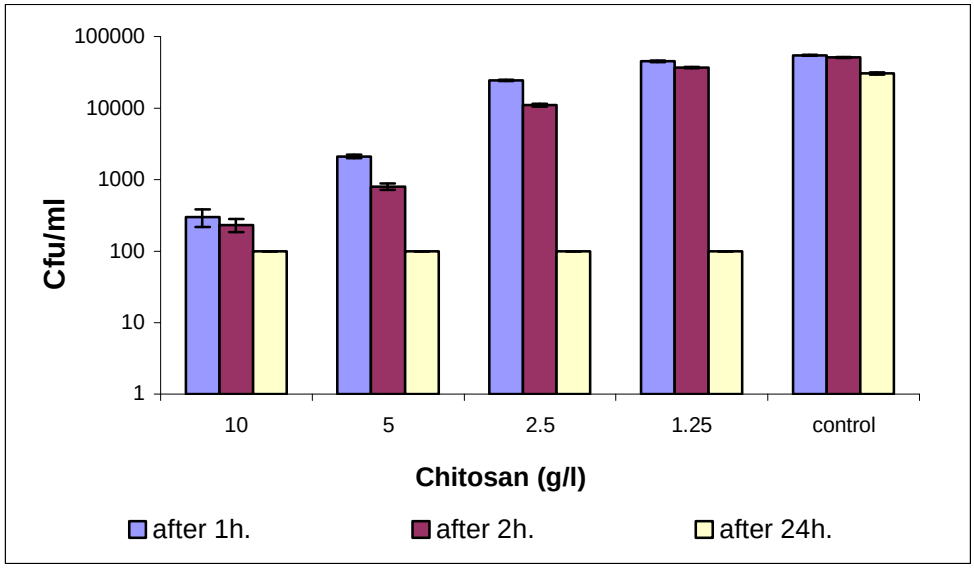


Figure 37: Effect of chitosan concentration on the control of *E.coli* at 37 °C, pH 6

3.2.3. Effect of chitosan MW on the reduction of bacteria

Samples of chitosan (CTS) of different MW and DD according to Table 7 were tested against *E.coli* and *Micrococcus luteus*. The effect of MW of CTS on the reduction of bacteria is described in Figure 38 and Table 14. For both types of bacteria, the antibacterial effect of dissolved CTS is more pronounced for the low MW chitosans. But at the same time it is obvious from the figures that the antimicrobial activity of CTS can not only attributed to its chain length. However, there is a complex balance of the chain length of the CTS molecules, their cationic nature, i.e. DD and the incubation time and it is this balance that plays an important role for the antimicrobial activity. The influence of MW of CTS on its antimicrobial activity was investigated in numerous publications [66]. Details about the effect of MW of chitosan on the antibacterial activity were discussed in the introduction chapter, section (1.3.9). The antimicrobial activity of CTS seemed to be mainly caused by blocking of nutrition supply through the cell wall of bacteria. Hwang et al. [61] concluded that CTS with MW about 30.000 Da exhibited the highest bactericidal effect on *E.coli* from their investigation of CTS MW range of 10.000-170.000 Da and this result agreed with the result in our study. All of these finding suggest that the antimicrobial activity of CTS can be related not only to its cationic nature but also to its chain length.

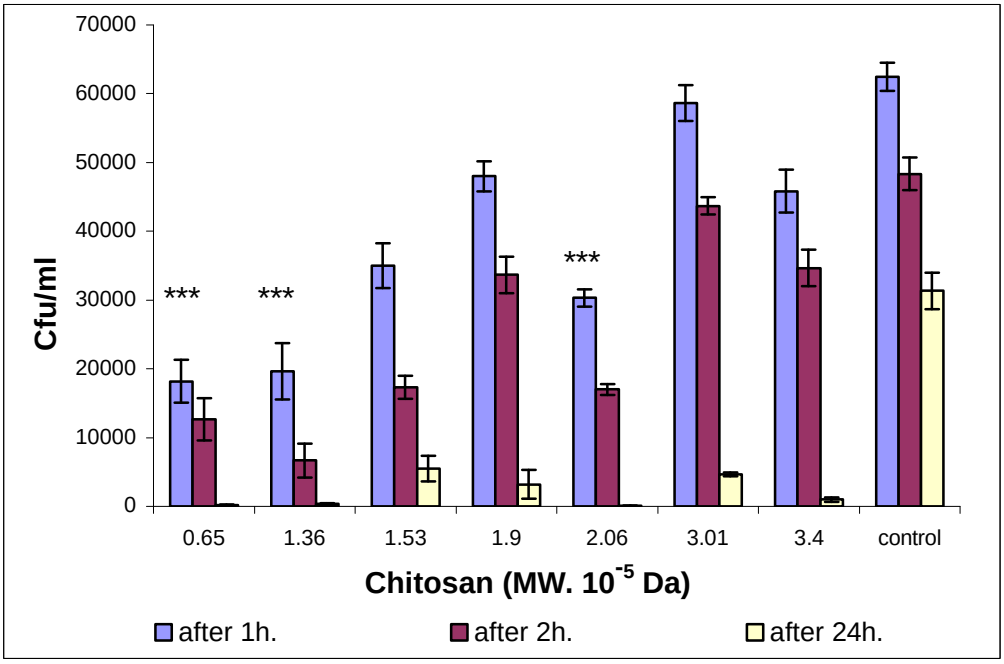


Figure 38: Effect of MW of chitosan on the reduction of *E.coli* at 25 °C, pH 6

Code	Pure	85/60	75/100	Bil 02	Bil 03	85/500	Bil 04	
(MW. 10^{-5})	**0.65**	**1.36**	**1.53**	**1.9**	**2.06**	**3.01**	**3.4**	**control**
Cfu/ml*10^2(1h)	9 ± 0.4	10 ± 1.6	12 ± 0.8	16 ± 0.8	11 ± 0.5	11 ± 0.8	29 ± 3.3	170 ± 32.7
Cfu/ml*10^2(2h)	5 ± 0.8	6 ± 0.8	8 ± 0.8	11 ± 1.3	7 ± 1.3	9 ± 2.1	13 ± 0.8	140 ± 8.2
Cfu/ml (24h)	= 100	= 100	= 100	= 100	= 100	= 100	= 100	103 ± 12.5

Table 14: Effect of MW of CTS on the reduction of *Micrococcus luteus* at 25 °C, pH 6

3.2.4. Effect of chitosan films on bacteria adhered to Molloplast® B-soft liner

The effect of chitosan films on the bacteria adhered to Molloplast® B was evaluated using the TTC-test method [144]. The activity of bacteria adhering to Molloplast® B soft liner coated with CTS films of different MW was reduced as described in Figure 39. The most active antimicrobial film was formed by the low MW chitosans like chitopure 21, He S 85/60 and Bil 03 (cf. Table 7). In Figure 39 the absorbance of formazan is directly proportional to the active adhering cells. The activity of the cells decreased approximately 10 times in comparison with the control groups. This clearly indicated the reduction of adhering cells by coating the soft liner with chitosan of low and intermediate MW.

It is really astonishing that immobilized CTS films used in our study showed a similar behaviour as the dissolved one. Samples of CTS of different MW and DD according to Table 7 were attached to Molloplast® B soft liner and the antimicrobial activity of the immobilized CTS films was tested against *E.coli*. Again the antibacterial effect of CTS increased by decreasing the MW of CTS (Figure 39).

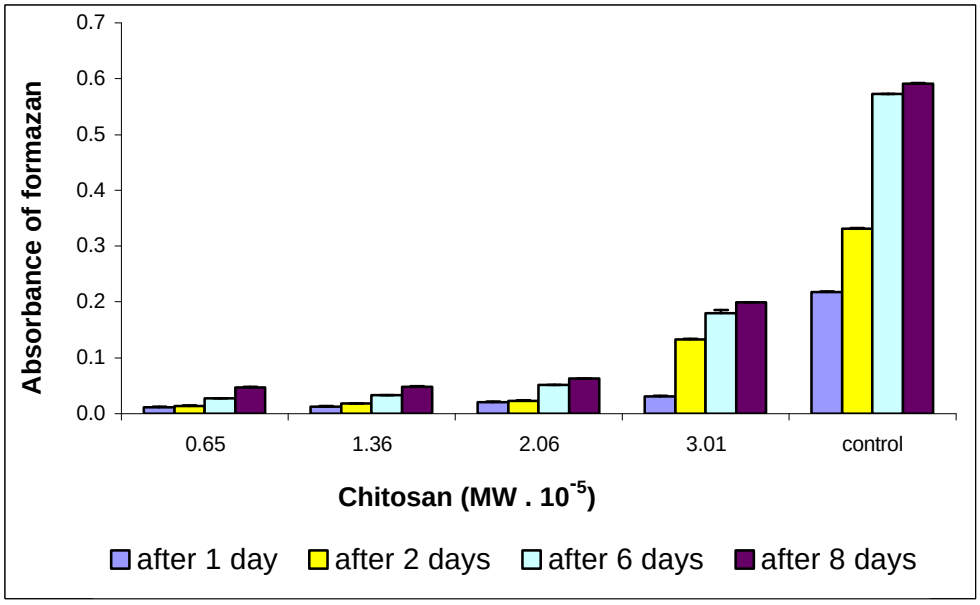


Figure 39: Effect of MW of chitosan films coated on Molloplast® B and incubation time on the reduction of bacteria at 37 °C, pH 6

3.3. Cyclodextrin in textile finishing

In this part ß-cyclodextrin (ß-CD) will be used as an exemplary model system for application in medical textile surfaces. In this part ß-CD will form complexes with some antimycotic agents. The drug release effect of ß-CD complexes against some types of fungi will be measured and evaluated by the laser nephelometry in microtiter plates method.

3.3.1. Introduction

To determine the *in vitro* susceptibility of fungal organisms to ß-Cyclodextrin (ß-CD) complexes with the antifungal agents Econazole nitrate (EC) and Ciclopirox-olamine (CI), a fast, rapid and simple method using laser nephelometry in 96-microtiter plate is used. The antimycotic influence of the complexes against *Candida albicans* DSM 11225 and *Candida krusei* ATCC 6258 species was determined using this method. In addition, the nephelometric method allows the determination of solubilities of active agents alone and in complexes.

Inclusion compounds (ICs) are composed of a host and a guest molecule. The host molecule surrounds and isolates the guest molecule during the co-crystallization process. The stability of these ICs depends on the physical proportions of the guest and host molecules, i.e. their stoichiometry and geometry. CD is a cyclic oligosaccharide that is able to form complexes with hydrophobic compounds by incorporating the compound, or more frequently some hydrophobic moiety of the compound into the ß-CD cavity [195]. No covalent bonds are formed or broken during the complex formation, and in solution unbound molecules may be in equilibrium with molecules bound in the complex [196,197]. The most common application of ß-CD in the field of pharmaceutical industry is to enhance drug solubility in aqueous solutions. In general, the lower the aqueous solubility of the pure drug, the greater the relative solubility enhancement gained by ß-CD complexation [196]. In this study the very low aqueous solubility of both antifungal agents EC and CI does not allow the preparation of a concentrated stock solution. Therefore, complexation with ß-CD improves the aqueous solubility of both drugs without modification of their original structures. This may allow a homogeneous delivery system of both antifungal agents and hence increase their bioavailability.

Antifungal azole derivatives are active *in vitro* and *in vivo* against a wide spectrum of pathogenic fungi. Econazole nitrate (1-[2-(2,4) dichlorophenyl)-2-(4-chlorobenzyloxy)-ethyl]- imidazole nitrate) (Figure 40) is such an antifungal agent. Its broad spectrum activity is demonstrated against most pathogenic agents responsible for human mycosis [198]. Microscopic investigations on bacteria and fungi treated with EC have shown alterations on the cellular and subcellular membranes [199]. Ciclopirox® olamine (CI) has a mechanism of action that is different from other antimycotic agents [200]. This antifungal compound is a synthetic hydroxypyridone derivative whose chemical formula is (6-cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridone) [201] (Figure 41). CI inhibits cellular uptake of essential compounds and at high concentrations can alter cell permeability [202,203]. CI also acts through chelating with polyvalent metal cations such as Fe^{3+} and Al^{3+} [204,205]. Chelating creates a larger, combined polyvalent cation, which has an inhibitory effect on enzymes that are involved in the cellular processes of fungi [203,206]. Pedersen (1993) studied the formation and antimycotic effect of cyclodextrin inclusion complexes of EC and Miconazole [207]. They found that the antimycotic effect of ß-cyclodextrin-econazole nitrate complex (CD-EC) against a strain of *candida albicans* was superior to the effect of a physical mixture of the two compounds.

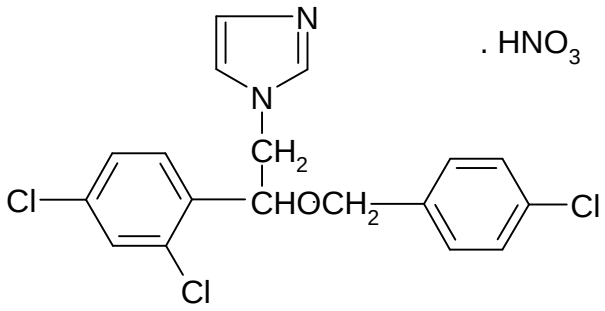


Figure 40: Chemical structure of Econazole nitrate

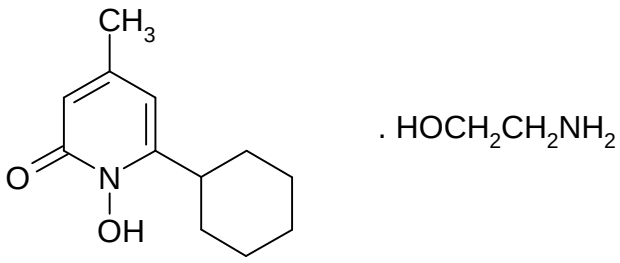


Figure 41: Chemical structure of Ciclopirox® olamine

3.3.2. Characterization of the prepared inclusion complexes

Stability constant

The stability constants of the two complexes were determined using the Potentiometric titrations method described as before in chapter 2. The obtained value of the stability constant for ß-cyclodextrin-econazole nitrate complex (CD-EC) was 2.92 ± 0.18 while the ß-cyclodextrin-ciclopirox olamine complex (CD-CI) has a value of 2.09 ±0.06.

Elemental analysis

The elemental analysis (C,H,N) of CD-EC and CD-CI complexes were determined. As a result, the molar ratio of the inclusion complexes were confirmed. The data assured that CD-EC complex was prepared in 1:1 molar ratio but the other complex between CI and CD was prepared in 1:2 molar ratio respectively as represented in the experimental section. The obtained data are summarized as following:

ß-CD: $C_{42}H_{70}O_{35}$
Calculated: C = 44.44 %, H = 6.17 %, O = 49.38 %
Found: C = 44.60 %, H = 6.34 %, O = 49.60 %

1:1 CD-EC complex : $C_{60}H_{86}Cl_3N_3O_{39}$
Calculated: C = 45.58 %, H = 5.44 %, N = 2.66 %, O = 39.50 %
Found: C = 45.72 %, H = 5.51 %, N = 2.61 %, O = 39.40 %

2:1 CD-CI complex : $C_{98}H_{164}N_2O_{73}$
Calculated: C = 46.33 %, H = 6.40 %, N = 1.10 %, O = 46.01 %
Found: C = 46.66 %, H = 6.17 %, N = 0.56 %, O = 46.62 %

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry is a thermal analysis technique which has already been used for several decades. It is applicable to a variety of materials including polymers, pharmaceuticals, foods and inorganics. DSC measurements provide qualitative and quantitative information as a function of time and temperature regarding transitions in materials that involve endothermic or exothermic process, or changes in heat capacity.

In this work CD-EC and CD-CI complexes were characterized by DSC to ensure that the inclusion complexes were formed. The obtained thermograms are shown in Figure 42. It could be seen (curves 1a,b) that the CD has a transition at 132 °C while each of EC (curve 3a) and CI (curve 3b) have a transition at 166 °C and 124 °C respectively, which were absent in both inclusion complexes (curves 2a,b). A small endothermic peak at 162 °C was recorded for CD-EC complex (curve 2a) which is due to fusion of very small amount of excess fine crystals of EC. The reduction of transition area of drug in that case assures the formation of the crystalline inclusion complex between CD and EC. This finding was in accordance with other related work [208]. Another exothermic peak was also recorded for CD-EC (curve 2a) which is due to oxidation of uncomplexed traces of drug.

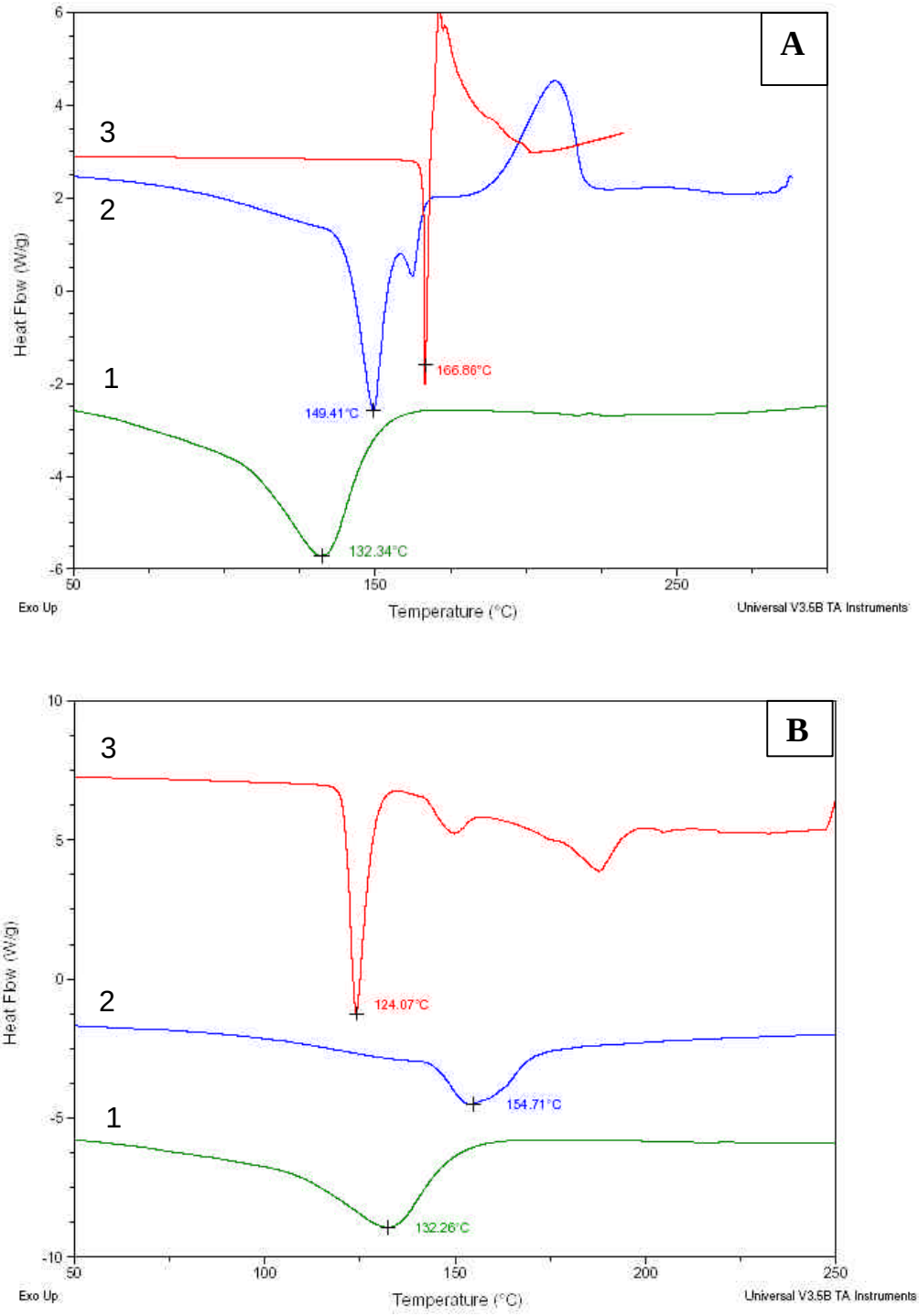
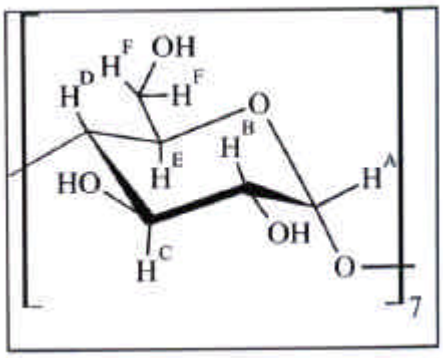


Figure 42: DSC curves of Econazole (A) and Ciclopirox (B) with ß-CD; 1, ß-CD; 2, Complex; 3, pure drug

^{1}H-NMR (DMSO-d_6, 500 MHz)



ß-Cyclodextrin (ß-CD): $C_{42}H_{70}O_{35}$;

d = 3.20 (*m*, 7H, H^D), 3.35 (two *d*., 7H, *J* = 6.4 Hz,H^B), 3.39 (*m*, 7H, H^E), 3.61 (*m*, 14H, H^F), 3.65 (*m*, 7H,H^C), 4.86 (*d*, 7H, *J* = 3.9 Hz, H^A).

Econazole nitrate (EC): $C_{18}H_{16}Cl_3N_3O_4$;

d = 4.34 (*d*, 2H, *J* = 7.5, N-CH_2), 4.58 (*m*, 1H, CH-O), 5.15 (*m*, 2H, O-CH_2), 7.20-7.75 (*m*, 10H, 7H in two Phenyl rings and 3H in imidazole ring).

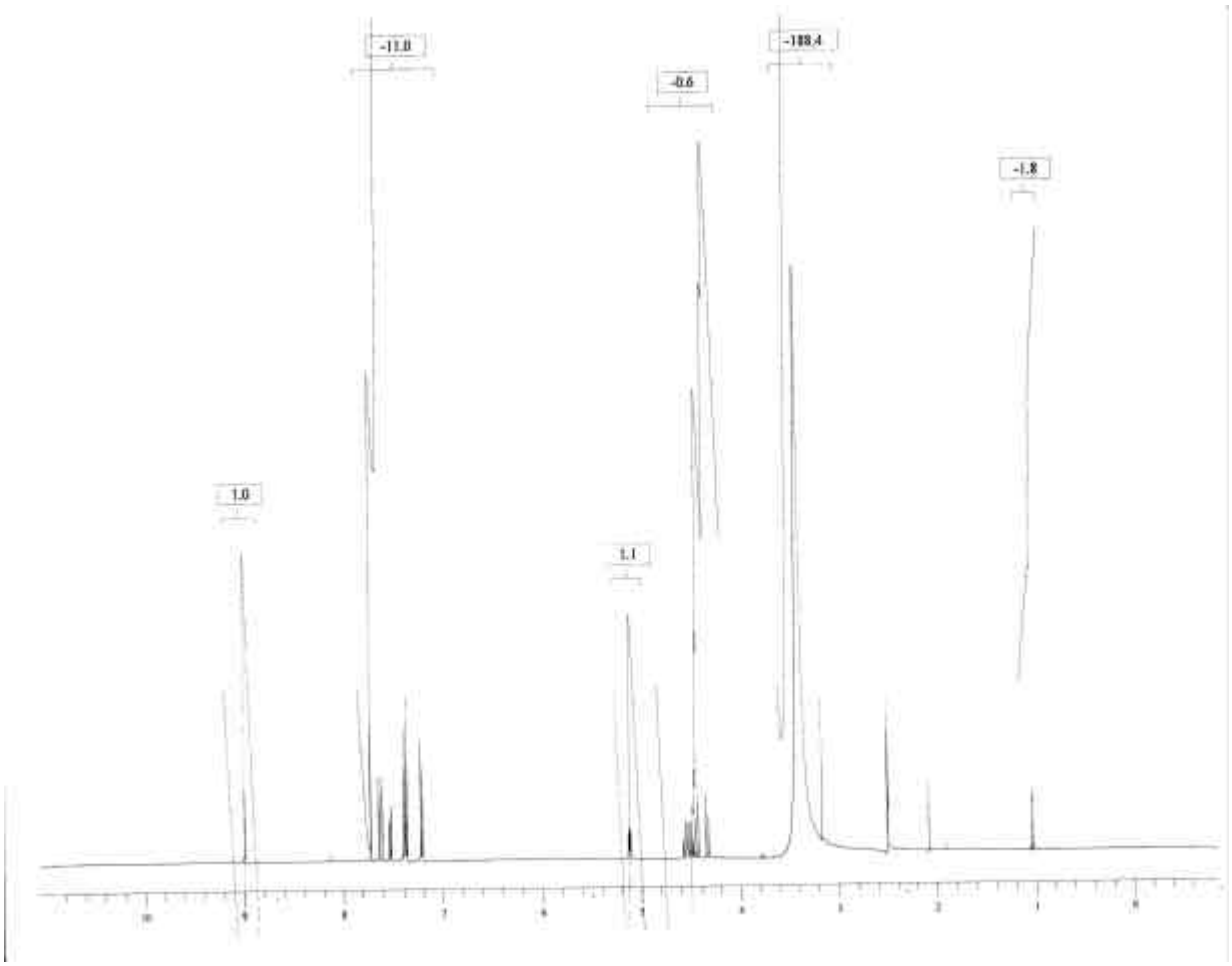


Figure 43: ^{1}H-NMR-spectra (DMSO-d_6, 500 MHz) of Econazole nitrate

ß-Cyclodextrin-Econazole nitrate complex (CD-EC): $C_{60}H_{86}Cl_3N_3O_{39}$;

d = 3.20 (*m*, 7H, H^D), 3.35 (two *d*., 7H, *J* = 6.4 Hz, H^B), 3.39 (*m*, 7H, H^E), 3.61 (*m*, 14H, H^F), 3.65 (*m*, 7H, H^C), d = 4.34 (*d*, 2H, *J* = 7.5, N-CH_2), 4.58 (*m*, 1H, CH-O), 4.86 (*d*, 7H, *J* = 3.9 Hz, H^A), 5.05 (*m*, 2H, O-CH_2), 7.20-7.62 (*m*, 10H, 7H in two Phenyl rings and 3H in imidazole ring).

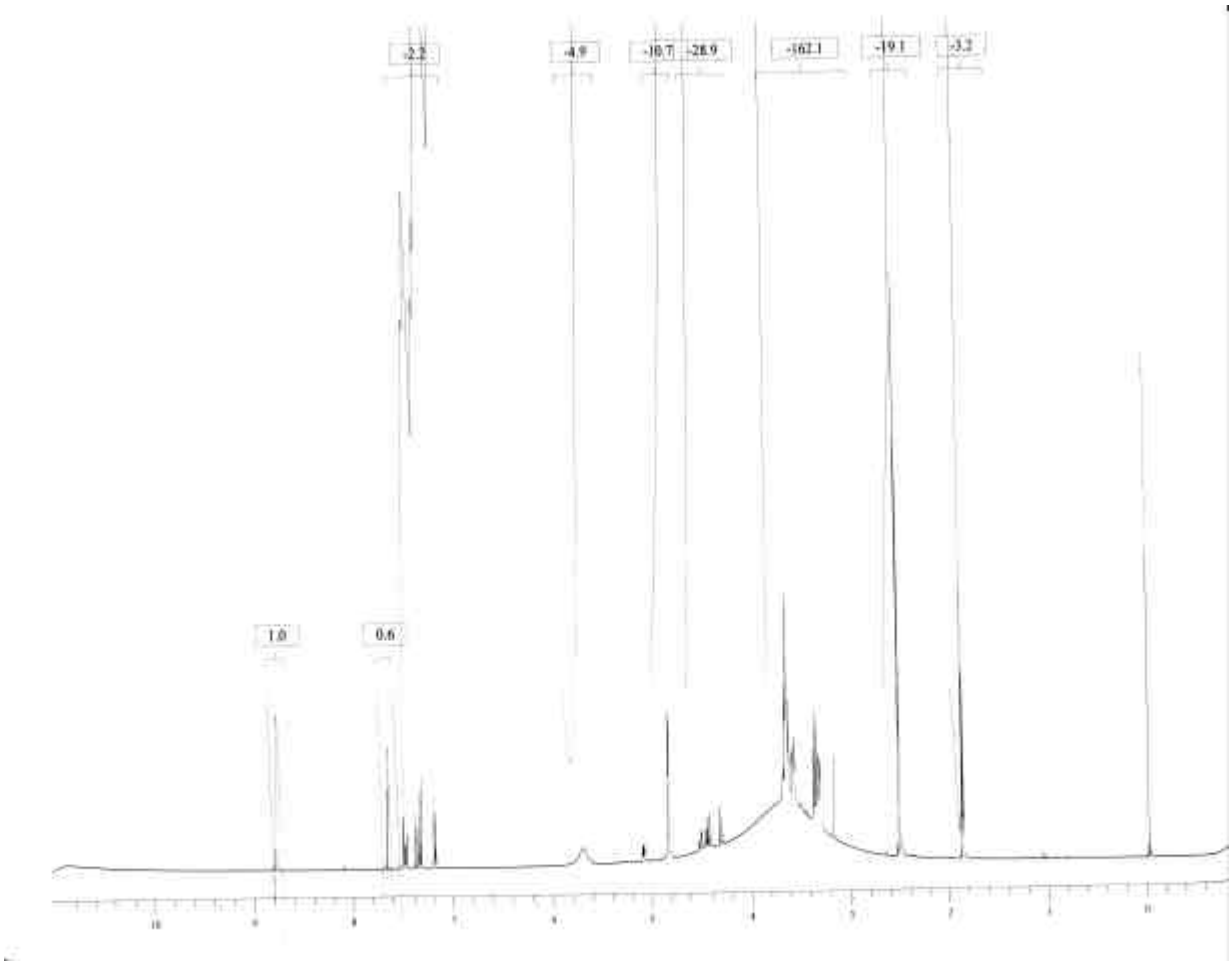


Figure 44: ^{1}H-NMR-spectra (DMSO-d_6, 500 MHz) of CD-EC complex

Phase solubility diagrams

Aqueous solubility of drugs is important to evaluate the bioavailability of compounds and eliminate the poorly soluble compounds showing good efficiency. Traditionally, “equilibrium” solubility has been determined by shaking the compound with the solvent of choice for at least 24 h or until no more will dissolve, then filtering, and determining the concentration of dissolved compound by a suitable analytical method. This approach is inappropriate in modern drug discovery setting for a number of reasons. For example, the weighing of hundreds of solid samples at submilligram quantities is no longer a viable proposition due to the time and manpower requirements. We describe a method based on laser nephelometry that can overcome these problems.

Solubility diagrams were monitored using laser nephelometry that can determine the solubility of potential drug candidates supplied as dimethyl sulfoxide (DMSO) solutions in 96-well plates [145]. The time required to determine the solubility of inclusion complexes varies widely from the usual period of 1-2 days to durations of 1-2 weeks [209]. Lipinski has published protocols for the determination of drug solubility by nephelometric and turbidimetric methods but the author does not control the percentage of DMSO cosovent in static microtiter plate wells [210]. In this study laser nephelometry overcame all problems of solubility. This system has been validated according to refs. [145,211]. All solubility measurements were carried out at 30°C, gain 122 and a laser intensity of 1 %. The concentration of DMSO co-solvent was constant at 5 % so the solubility enhancement by a gradual increase in DMSO is avoided. It is clear from Figure 45 and 46 the inflection point at which precipitation of the drug takes place. In the absence of ß-CD, the solubility of EC and CI was determined to be ~ 3.9 mg/ml and ~ 1.2 mg/ml, respectively, while in the presence of ß-CD the solubility increased up to ~ 16 mg/ml for CD-EC complex. Linear curve was obtained for CD-CI complex and no point of precipitation was found which indicated complete solubility over the concentration ranges as described in Figure 45, Figure 46 (A,B).

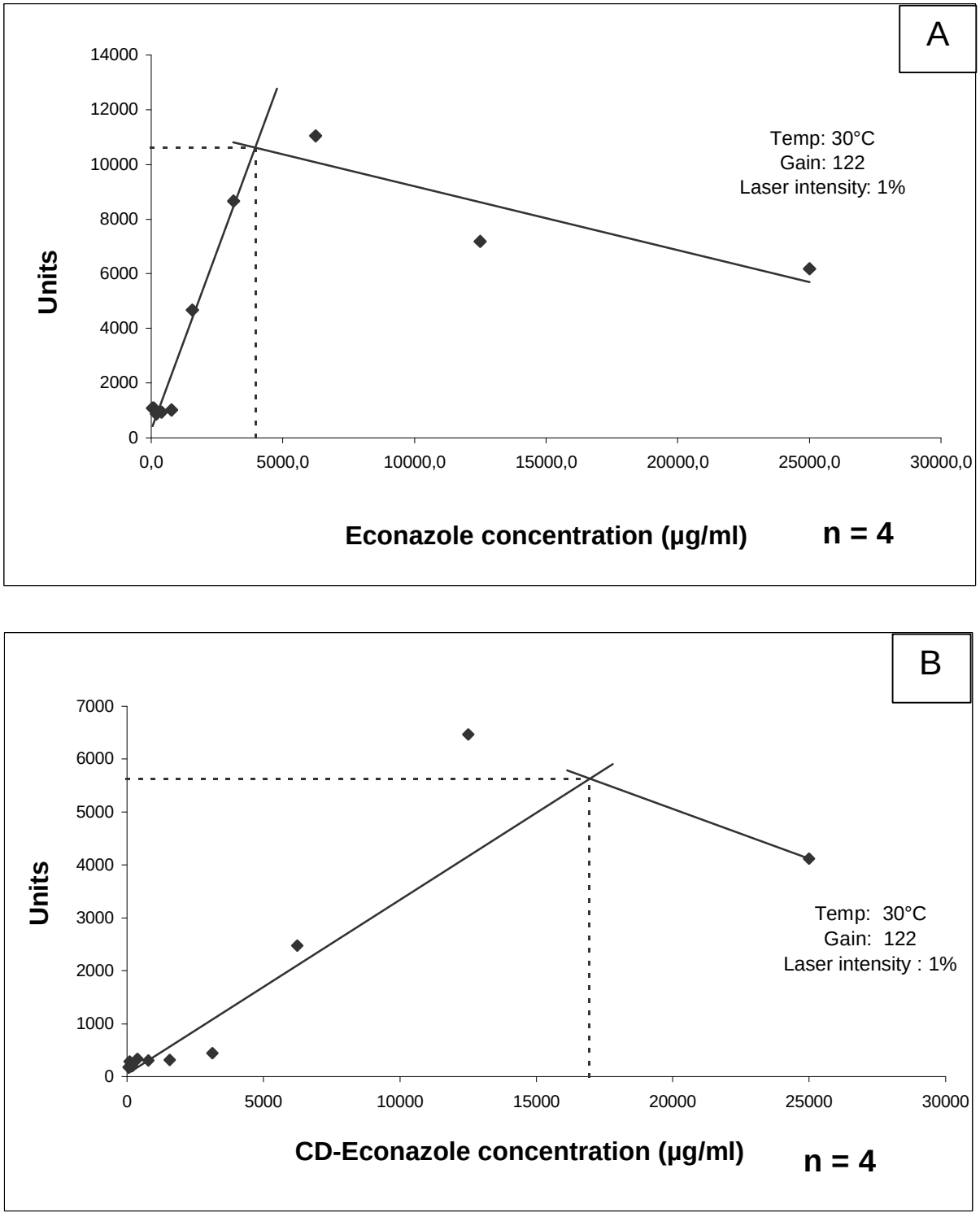


Figure 45: Solubility diagram of (A) Econazole and (B) CD-Econazole complex (determined by using the laser nephelometry soft ware)

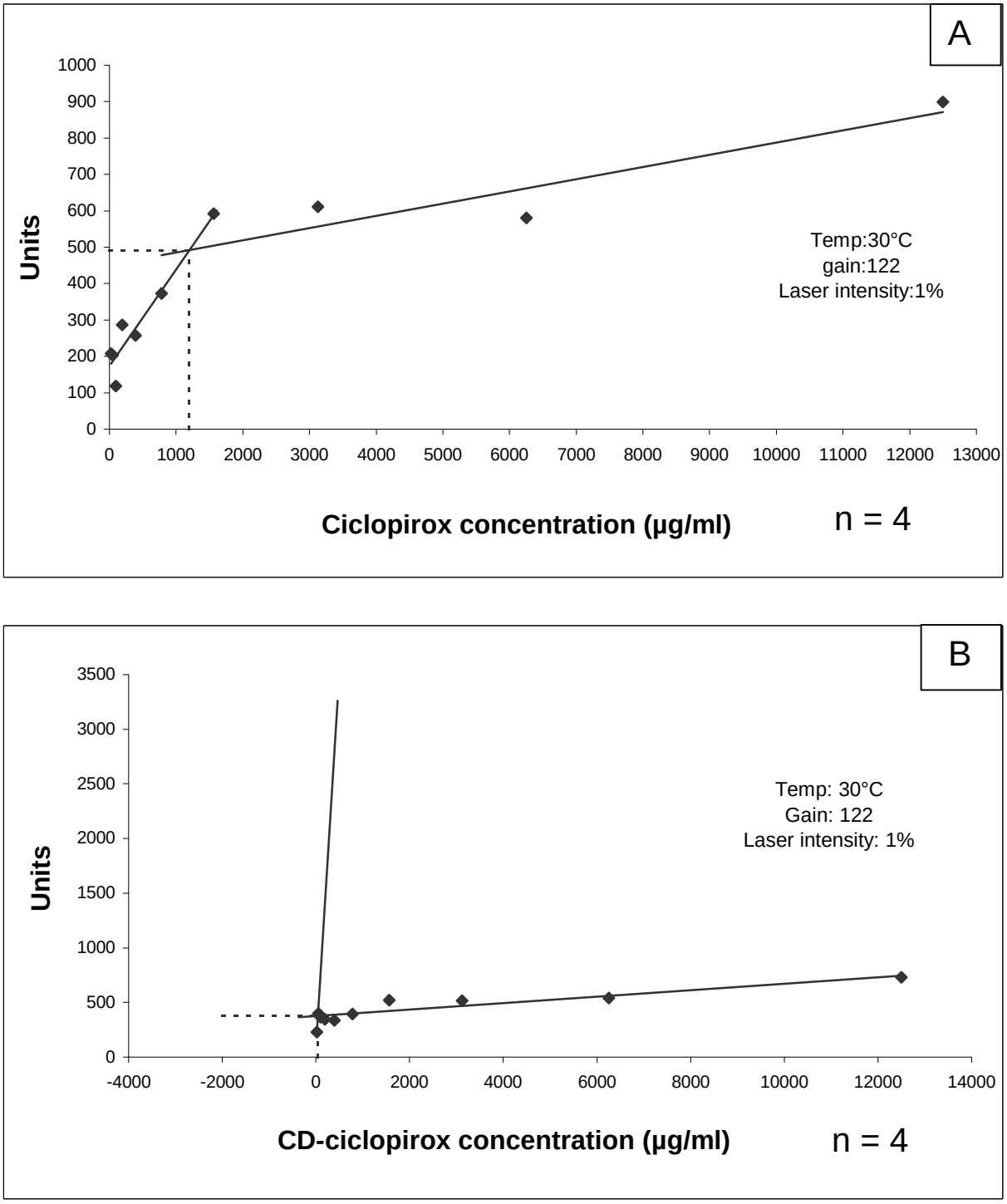


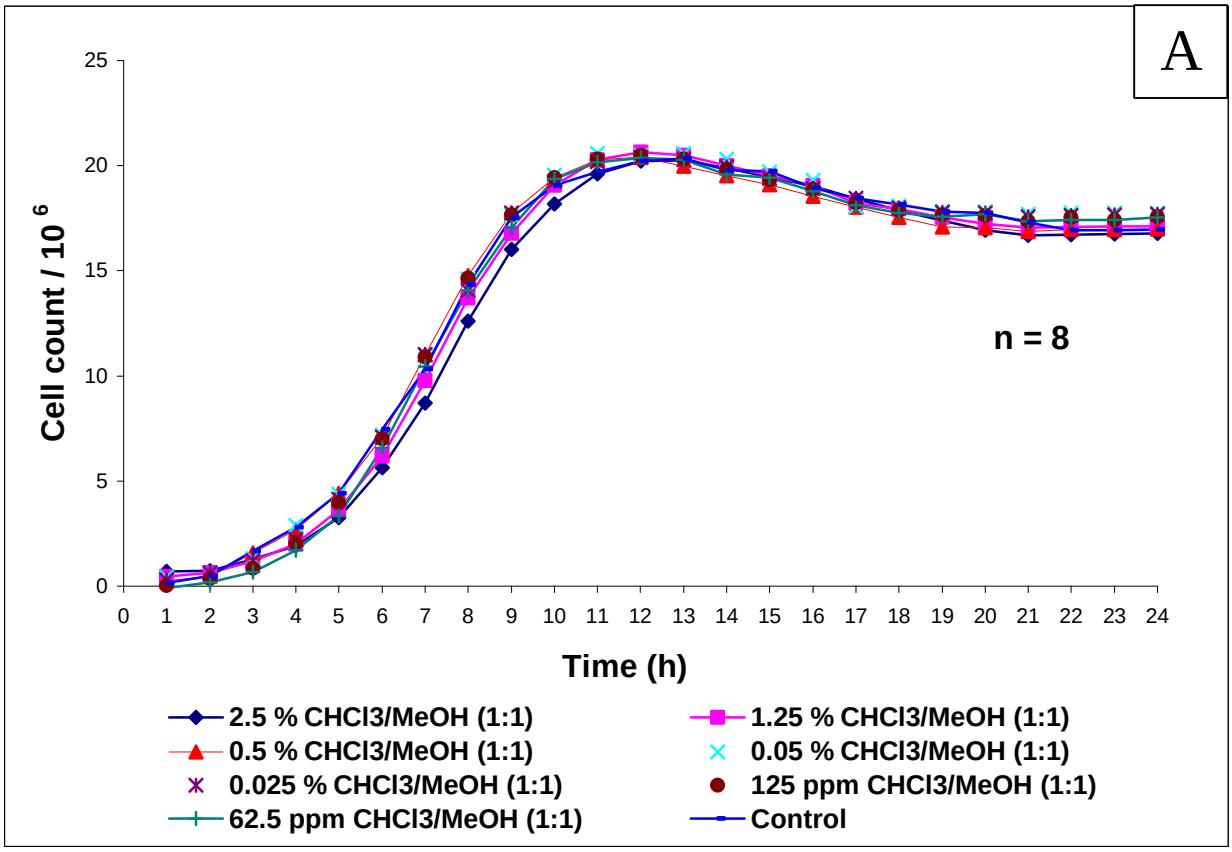
Figure 46: Solubility diagram of (A) Ciclopirox and (B) CD-Ciclopirox complex (determined by using the laser nephelometry soft ware)

3.3.3. Factors affecting the *Candida* species cell growth

All factors affecting the *candida* species cell growth were studied and evaluated using laser nephelometry. Given below are the results obtained along with appropriate discussion.

3.3.3.1. Influence of solvent

The influence of a mixture of chloroform/methanol (1:1) as negative control on the cell growth of both *Candida* species was evaluated to know whether this solvent mixture has an effect on *Candida* species or not. It was found clearly that for all concentrations used ranged between (2.5 %- 62.5 ppm), no significant effect of this solvent on the cell growth of both organisms in comparison with control was found since the growth curve was not disturbed, as described in Figure 47 (a,b).



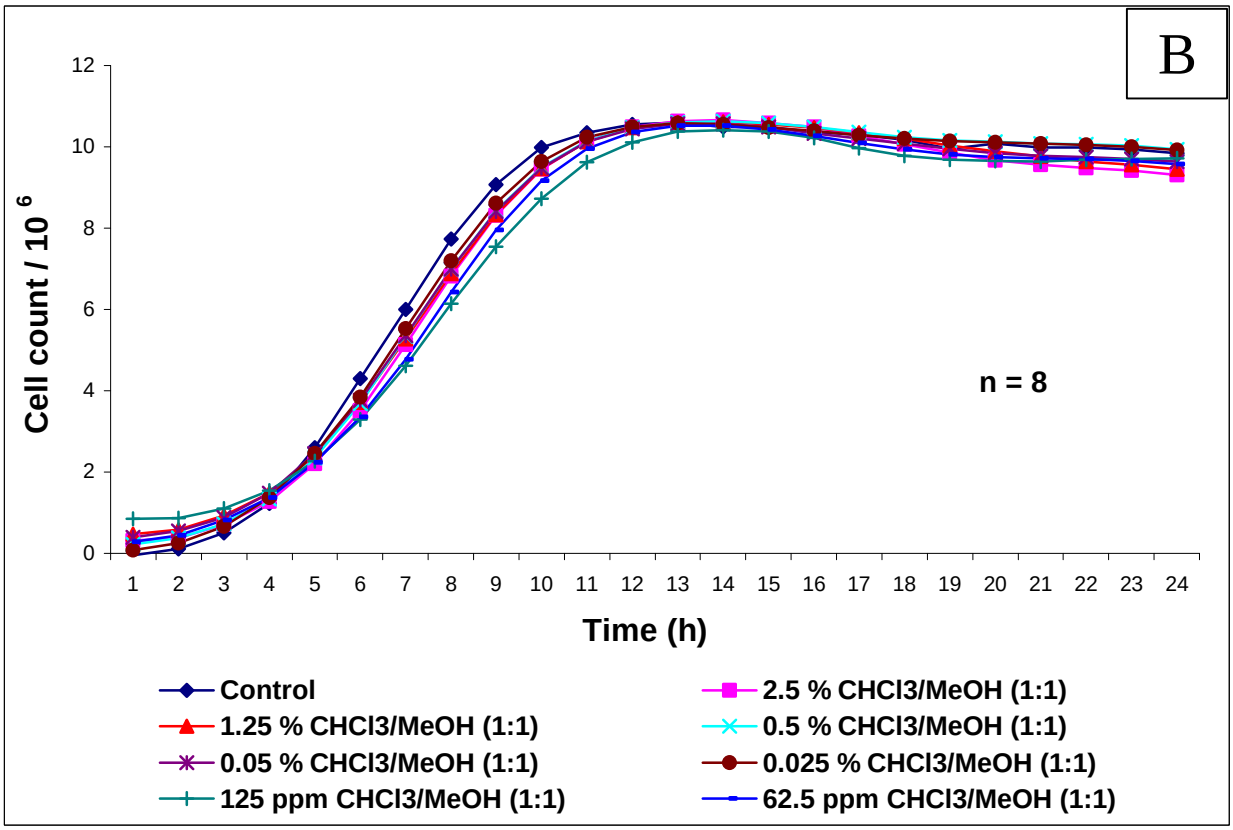


Figure 47: Influence of Chloroform/Methanol mixture $CHCl_3$ / MeOH 1:1 on: (A) *Candida albicans* DSM 11225; (B) *Candida krusei* ATCC 6258

3.3.3.2. Influence of Econazole nitrate

The data represented in Figure 48 (a,b) demonstrate that EC has more pronounced effect against *C.Krusei* than against *C.albicans*. With respect to *C.albicans* a concentration of 25 µg/ml of EC inhibited the cell growth while at 50 µg/ml cells were killed in comparison with control. On the other hand, using a concentration of 10 µg/ml of EC inhibited the cell growth of *C.Krusei* while at 12.5 µg/ml cells were completely killed. Inhibition of the cell growth by EC can be explained either by metabolism of EC or by sequestration of this product by cell constituents like proteins or lipids [212]. A preliminary study with ^{125}I labelled EC according to the Greenwood and Hunter method showed penetration of this product into the cells [213].

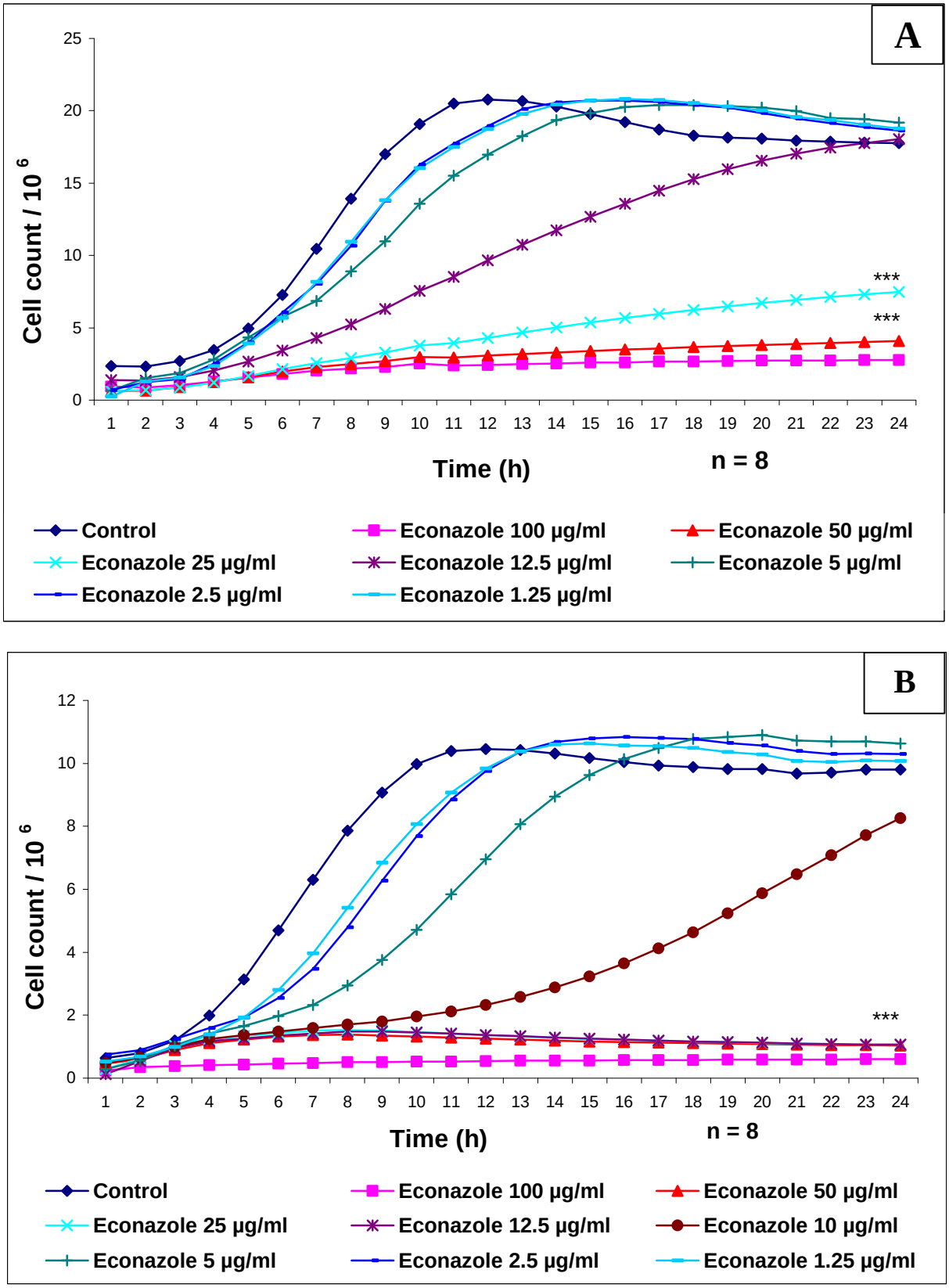
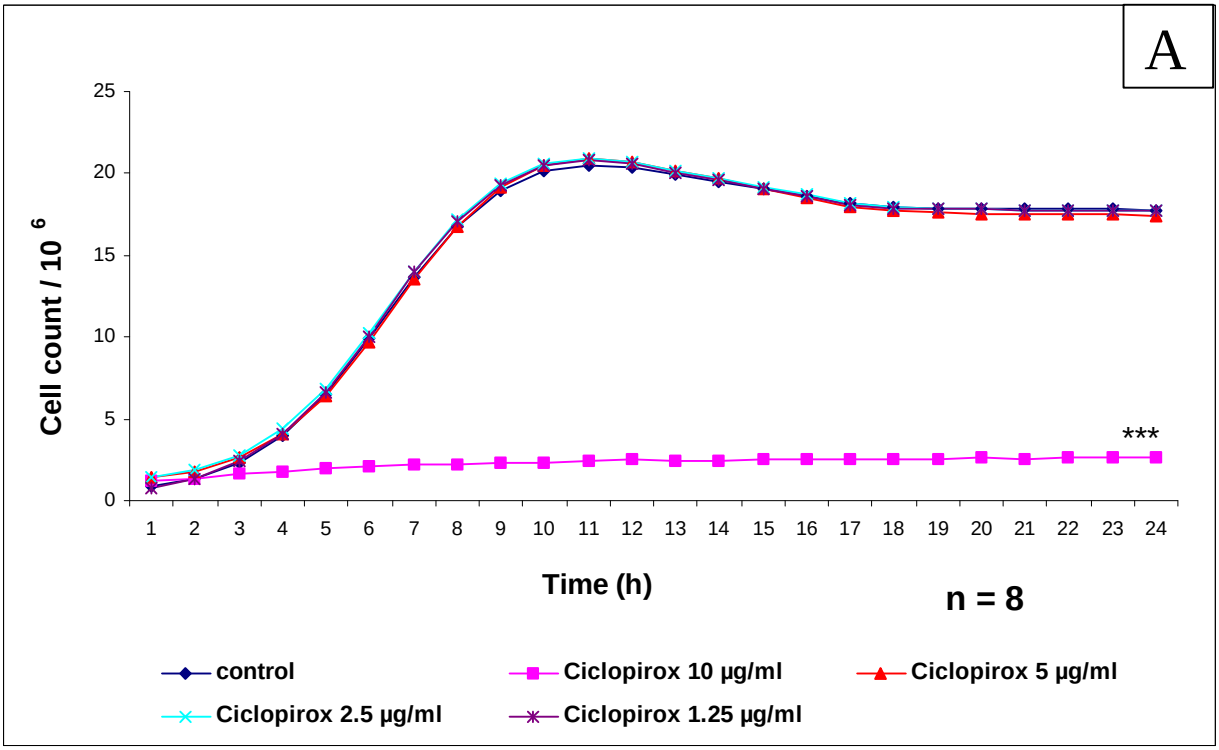


Figure 48: Influence of Econazole nitrate on:

(A) *Candida albicans* DSM 11225 ; (B) *Candida krusei* ATCC 6258

3.3.3.3. Influence of Ciclopirox-olamine

Ciclopirox olamine (CI) is effective against a broad spectrum of fungal organisms including dermatophytes, yeast and moulds acting in a fungistatic or fungicidal manner in vitro [203,214,215]. CI shows a greater in vitro activity towards *C.albicans* than other antimycotics tested [204]. CI has also shown in vitro activity against *Malassezia furfur* [216,217]. In our study CI was more effective against *C.albicans* at very low concentration (10 µg/ml) where all cells were killed while for other concentration ranges from 1.25 to 5 µg/ml the cells were more active as described in Figure 49 (a,b). This finding was in agreement with the above previously mentioned results. For *C.krusei,* the drug was less effective than with other organism. At 12.5 µg/ml cells were killed and at other concentration less than 12.5 µg/ml cells were more active. CI was found to have the highest in vitro activity against both fungi and bacteria when compared to EC [216].



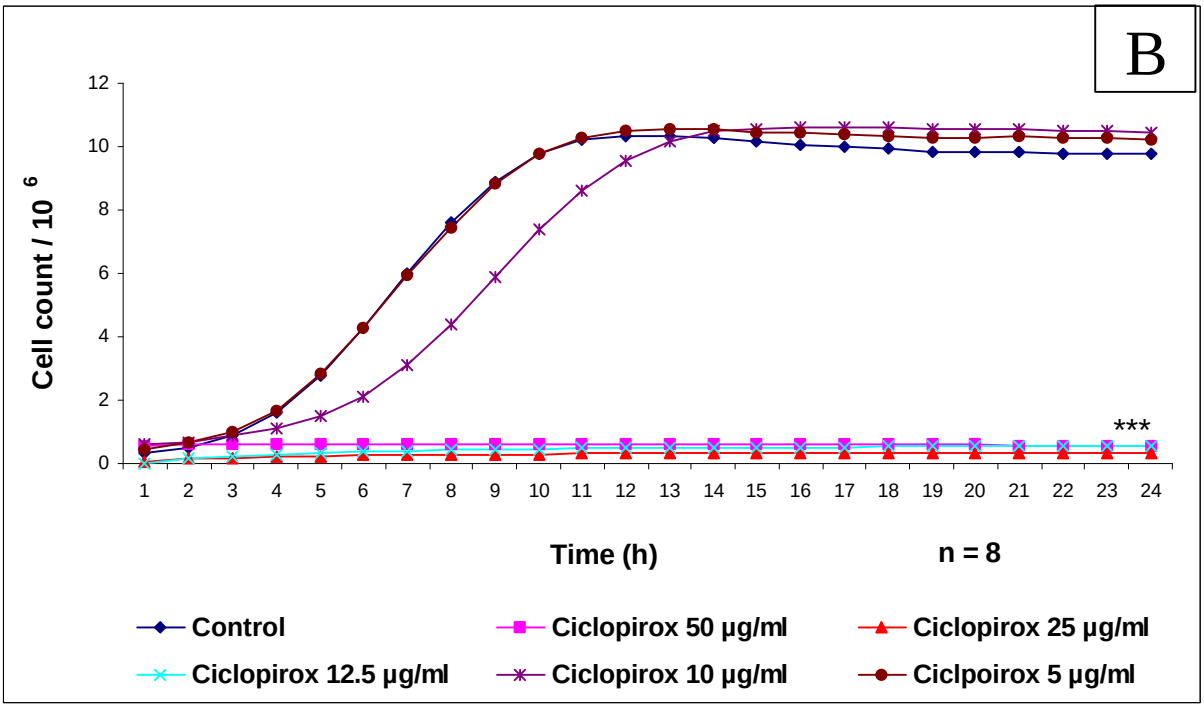
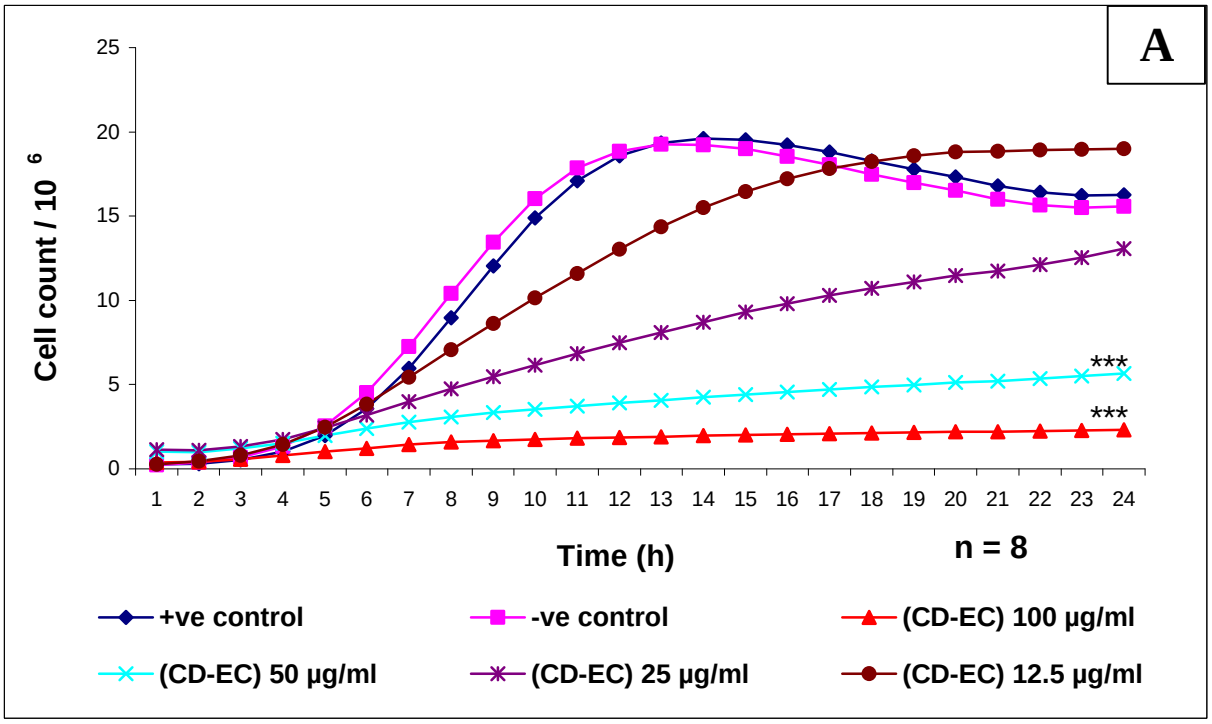


Figure 49: Influence of Ciclopirox-olamine on:
(a) *Candida albicans* DSM 11225 ; (b) *Candida krusei* ATCC 6258

3.3.3.4. Influence of ß-CD-Econazole complex

Econazole nitrate (EC) is an antimycotic drug used both locally and systematically. Improvement of solubility and release rate by complexation with ß-CD is therefore essential for rapid antimycotic activity [208]. Figure 50 (a,b) shows the results obtained by EC. *Candida albicans* was inhibited at a concentration of 25 µg/ml of CD-EC complex, while cells were killed at 50 µg/ml of the same complex in comparison with control. *Candida krusei* was inhibited at a concentration of 12.5 µg/ml of the complex and killed at 25 µg/ml. By using laser nephelometry in microtiter plates as a new method, the antimycotic influence was studied every hour until 24 h. In all growth phases, especially the logarithmic and stationary phases, the results obtained were significant and reproducible for the concentration ranges used either for drug alone with the challenge organism or with ß-CD complexes. In other study the antimycotic effect of the saturated drug solutions containing various cyclodextrin concentrations was estimated on the basis of inhibition zone size [207,208]. The largest inhibition zone for growth of *Candida albicans* was obtained in case of inclusion complex of the drug with ß-CD. This finding was attributed to the higher dissolution rate of this inclusion complex as compared to the drug alone in correlation with the solubility data, and

these reflect the higher antimycotic activity by rapid diffusion through agar medium. These finding results were in accordance with the results in this study.



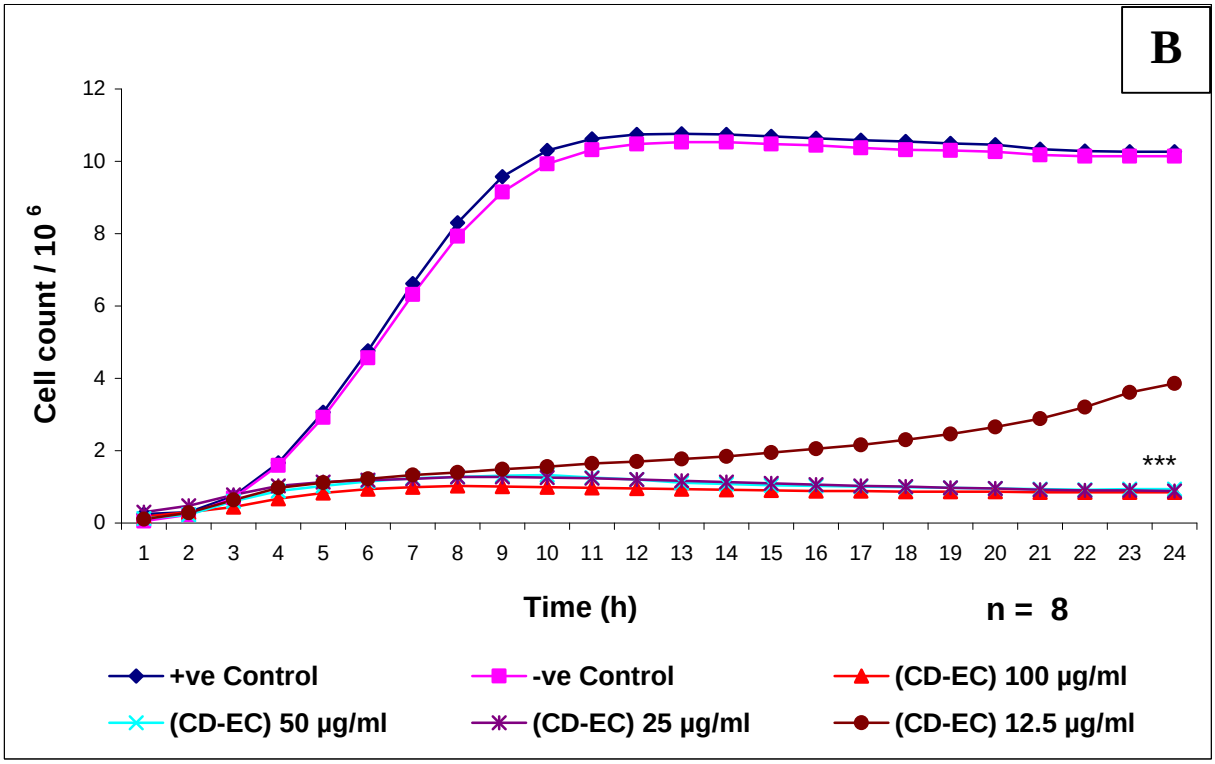
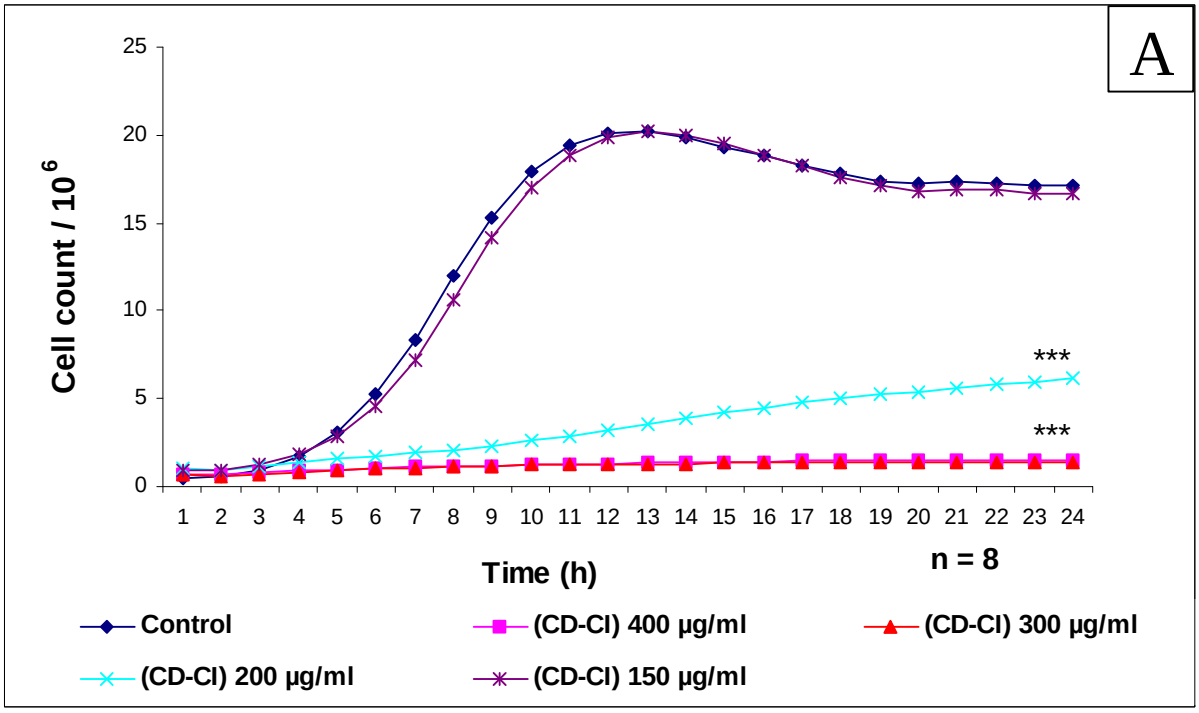


Figure 50: Influence of ß-CD-Econazole nitrate complex on :
(a) *Candida albicans* DSM 11225 ; (b) *Candida krusei* ATCC 6258

3.3.3.5. Influence of ß-CD-Ciclopirox complex

The efficiency of complexation may sometimes be rather low, and therefore relative large amounts of ß-CD must be used to complex small amounts of drug [196, 218]. The particle size of solid ß-CD complexes can affect the dissolution rate and therefore the bioavailability of the product. The particle size distribution and crystalline properties of the complex are dependent on the method of complex preparation [219]. In this study, ß-cyclodextrin-ciclopirox olamine complex (CD-CI) was less effective against *C.albicans* than other complex. At concentration of 200 µg/ml of the complex, cells were inhibited but at 300 µg/ml of the same complex cells were killed as described in Figure 51. As for *C.krusei* the complex was more effective. At a concentration of 200 µg/ml cells were killed and for other concentration less than 200 µg/ml cells were active.



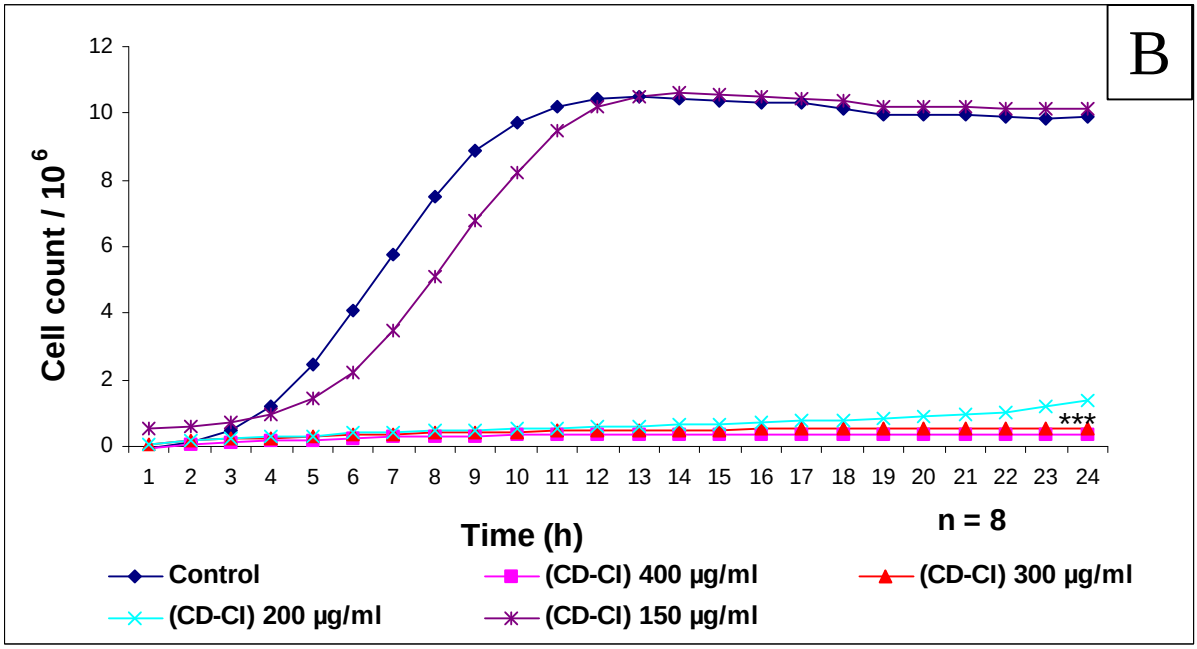


Figure 51: Influence of ß-CD-Ciclopirox-olamine complex on:
(A) *Candida albicans* DSM 11225 ; (B) *Candida krusei* ATCC 6258

3.3.4. Metabolic activity evaluation by vital staining with Fun-1

The widespread use of fungi to mediate biotransformation of organic materials has promoted the development of numerous techniques for measuring the viability and metabolic activity of these organisms [220]. There is appreciable variation in the speed, simplicity, sensitivity and reliability of techniques that are currently in use for this purpose. Conventional direct count methods, which typically involve vital staining with indicators such as methylene blue or tetrazolium salts, are used to assess the activity of cellular oxidoreductases in fungi [221]. Although this assay is simple to perform, it has been shown to be unreliable for fungi because of inconsistencies in the permeability or retention of the dyes, as well as the non-specific surface labelling of these cells [222].

A higher degree of sensitivity may be achieved with a unique new group of indicators that generate differential staining patterns in live and dead cells and do not require the living cells to be in an actively dividing state. The FUN-1 stain is representative of this class of indicators.

FUN-1 is a new family of fluorescent probes that has been developed for assessing the viability and metabolic activity of fungi. FUN-1 stain is a halogenated unsymmetrical cyanine which is virtually nonfluorescent in aqueous solution. However, after complexation with DNA or RNA and excited with light between 470 and 500 nm, FUN-1 stain fluorescence at 530 nm increased as much as 400-fold [223]. This class of dyes is exemplified by the FUN-1 stain: [2-chloro-4-(2,3-dihydro-3-methylbenzo-1, 3-thiazol-2-ylidene)-methyl-1-phenylquinolinium] iodide. A membrane-permeant nucleic acid-binding dye that has been found to give rise to cylindrical intravacuolar structures (CIVS) in fungi [221]. Biochemical processing of the dye by active cells yielded CIVS that were markedly red shifted in fluorescence emission and therefore spectrally distinct from the nucleic acid-bound form of the dye as described in Figure 52.

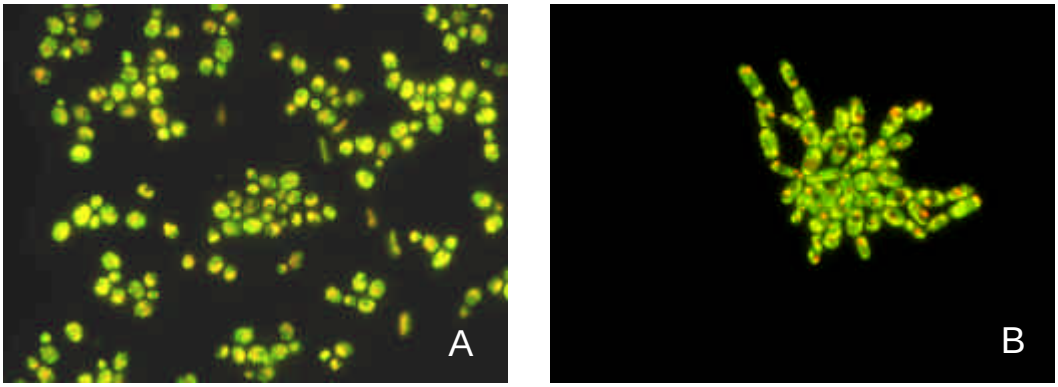


Figure 52: *Candida albicans* (A) and *Candida krusei* (B) (Control) stained with FUN-1 dye and observed by fluorescence microscope

The antimycotic influence of ß-CD-drug complexes can not be evaluated without consideration of the morphology of *Candida* species [224]. Also FUN-1 staining helps to characterize azole resistant strains [225,226]. In this study the metabolic activity of the cells was demonstrated by vital staining with FUN-1 resulting in red fluorescent with CIVS structures in both *Candida* species in the controls (Figure 52), which indicated the activity and viability of these cells. But under the influence of CD-EC and CD-CI complexes, cells were green or pale yellow fluorescent. That means, most of the cells lost their metabolic activity. Different images for cells stained with FUN-1 affected by each complex in different inhibitory (Figure 53,55) and lethal concentrations (Figure 54,56) are described.

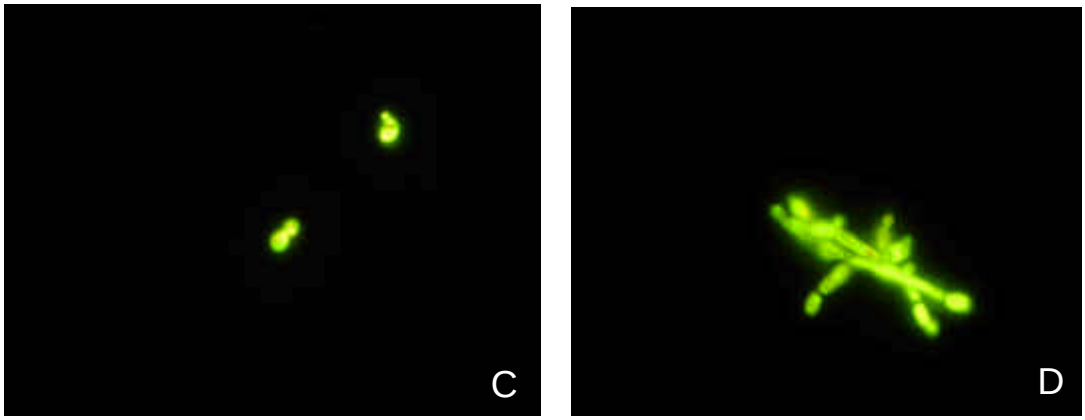


Figure 53: Metabolic activity of (CD-EC) complex (inhibitory conc.) against *C.albicans* (C) and *C.krusei* (D); stained with FUN-1 dye

Figure 54: Metabolic activity of CD-EC complex (lethal conc.) against *C.albicans* (E) and *C.krusei* (F); stained with FUN-1 dye

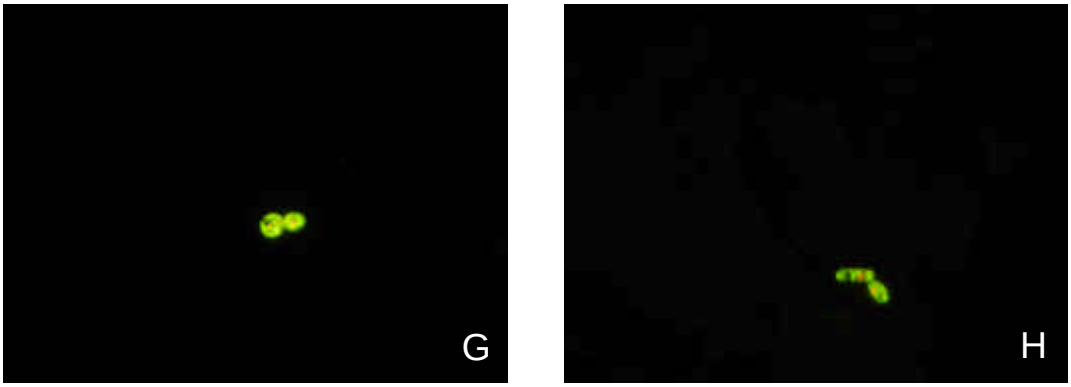


Figure 55: Metabolic activity of CD-CI complex (inhibitory conc.) against *C.albicans* (G) and *C.krusei* (H); stained with FUN-1 dye

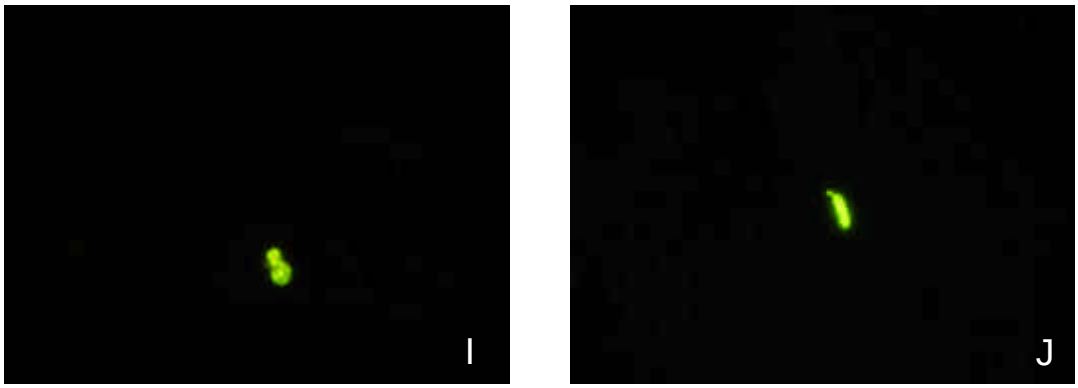


Figure 56: Metabolic activity of CD-CI complex (lethal conc.) against *C.albicans* (I) and *C.krusei* (J); stained with FUN-1 dye

Summary

According to the results in this thesis the antimicrobial activity of chitosan was evaluated quantitatively against both bacteria and fungi by using different methods; quantitative suspension method and laser nephelometry in microtiter plate. Chitosan samples, of different molecular weight and degree of deacetylation were dissolved in acetic acid 1 %. The pH of these samples were adjusted at pH 6. The antimicrobial activity of dissolved chitosan was measured and evaluated. Chitosan was used successfully as antimicrobial finishing agent in medical textile applications. Different anchors namely cyanuric chloride, 2,4-dichloro-6-methoxy-s-triazine, propane tricarboxylic acid and butanetetracarboxylic acid were used for the grafting of chitosan onto cotton fabrics. The grafting yield by weight % was determined. The antibacterial activity of the finished fabrics were evaluated by using the tetrazolium/formazan test method (TTC). From the results it was found that, as the anchor concentration increased, the grafting yield of chitosan increased significantly. Butanetetracarboxylic acid was a more pronounced anchor for the grafting of chitosan onto cotton fabrics than any other anchors used, because the grafting yield of chitosan increased up to 13 % by weight. Also the antibacterial activity of the finished cotton using this anchor was enhanced by increasing the grafting yield of chitosan. However, by using such polycarboxylic anchors to fix chitosan onto cotton leads to loss of physical and performance properties of cotton fabrics.

To overcome this problem, ionic crosslinking of cotton was carried out. In order to solve problems associated with chitosan such as limited solubility, antimicrobial activity and poor laundering durability when applied to cotton fabrics, a water soluble chitosan derivative, namely carboxymethyl chitosan was successfully prepared from chitosan and monochloroacetic acid in presence of alkali. The water soluble product was characterized by FTIR and solid state ^{13}C-n.m.r. spectroscopy. Cationization of cotton was carried out using 2,3-epoxypropyltrimethyl ammonium chloride (Quab® 151) in the presence of alkaline aqueous solution. Then, carboxymethyl chitosan was applied to the pre-cationized cotton using cold-pad-batch method. As a result of this treatment ionic crosslinked cotton was obtained. The treated cotton fabrics showed significant increases in crease recovery angles without strength loss. Also the wettability of the treated fabrics increased significantly. The antibacterial activity of the ioni-

cally crosslinked cotton increased also, but the antibacterial efficiency was not higher when compared with the previously chitosan-treated cotton using the polycarboxylic anchor group. So, ionic crosslinking of cotton improved the physical and performance properties of cotton fabrics combined with a moderate antibacterial activity.

Chitosan was also applied in the field of modern dentistry. Chitosan was successfully used as antimicrobial coating agent on Molloplast® B- soft liner based material for dentures to treat the so called denture stomatitis. Different types and molecular weights of chitosan-films (1 % in acetic acid 1%) were applied to the Molloplast® B. The integrity and durability of each film was established. The antibacterial activity of each film was evaluated by the TTC-test method. It was found from the results that coating the Molloplast® B soft liner with chitosan films reduces significantly the colony growth of microorganisms. The antimicrobial activity of chitosan-films increases with decreasing molecular mass of the biopolymers. It is concluded from the results that chitosan can be used as a powerful antimicrobial bio-adhesive material to reduce denture stomatitis.

The *in vitro* sensitivity of fungal organisms to ß-cyclodextrin complexes with the antifungal agents econazole nitrate and ciclopirox olamine has been determined using laser nephelometry in 96-well microtiter plates. This method has been shown to be a reliable technique for the measurement not only for the drug solubility but also for the antimycotic influence of drug, whether alone or in complex with ß-cyclodextrin. From the results obtained, a rapid inhibition and even killing of both fungi is observed only above certain concentration of complexes ranging between 12.5-100 µg/ml for ß-cyclodextrin-econazole nitrate complex, while it was in the range of 150-400 µg/ml for the ß-cyclodextrin-ciclopirox olamine complex. The stability constants of the two complexes were measured. Also the improvement of solubility of both antimycotic agents in PBS buffer was observed by complexation with ß-cyclodextrin. This study has proven that laser nephelometry in 96-well microtiter plates can be used as an efficient method for rapid determination of the solubility of potential drug compounds. Laser nephelometry can distinguish between the concentration at which the drug just dissolves or just separates out of solution. In addition, this technique can be used efficiently for monitoring and evaluating the growth phases of micro-organisms like fungi or bacteria.

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Curriculum Vitae

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May 1995	Bachelor of Science (BSc in Chemistry), (Very good with Honour degree), Chemistry Department, Faculty of Science, Mansoura University, Egypt.
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Publications

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Statement

I declare that I wrote this thesis myself. I did not use auxiliary materials other than indicated. Other's work was always cited. This thesis was never submitted to departments of other universities.

Krefeld, 17. Mai 2005 ***(Moustafa M. G. Fouda)***